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PEROXISOMAL AND MITOCHONDRIAL ENZYMES INVOLVED IN LIPID METABOLISM – STUDIES ON FUNCTION AND REGULATION

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ABSTRACT

Fatty acids constitute a major part of the energy that we obtain from the diet and are also the principal source for mammals to store energy. To use the incoming or stored fatty acids as energy, the fatty acids needs to be metabolized of which the majority of fatty acids will be degraded by the mitochondrial β-oxidation system that in the end generates energy to the cell in the form of ATP. However, this organelle is not able to handle all kinds of fatty acids of which very long chain fatty acids, long chain methyl-branched fatty acids and dicarboxylic acids are such cumbersome fatty acids. Therefore a second organelle, the peroxisome, is required for metabolism of these particular fatty acids. Also peroxisomes contain a β-oxidation system and similar to the mitochondrial system is the initial substrate a CoA-esterified fatty acid, so-called acyl-CoA.
This thesis will focus on some enzymes that are active on these acyl-CoA esters, but that are not directly involved in the β-oxidation per se. Instead they contribute to the regulation of both acyl-CoA and free coenzyme A levels in different cellular compartments. This thesis will also include how these fatty acid degrading systems can be regulated at gene level by affecting different transcription factors by dietary ligands and by fasting.

The peroxisomal Nudix hydrolase 7α (NUD7α), previously believed to be a CoASH degrading enzyme, was demonstrated to be a medium chain diphosphatase, most active on medium chain acyl-CoA esters, to produce 3',5'-ADP and the corresponding 4'-acylphosphopantetheine thereof. NUDT7α expression and activity was down regulated by PPARα activation, which would prevent CoASH degradation and support a high rate of the β-oxidation in peroxisomes during these conditions.

Peroxisomes are not only needed for the degradation of complex lipids, but are also essential for many other metabolic pathways such as bile acid and etherphospholipid synthesis and the degradation of D-amino acids and glyoxylate. The expression of gene transcripts that code for the proteins involved in these peroxisomal pathways was investigated almost throughout the whole mouse body with the aim to map the tissue expression of these pathways. The peroxisomal β-oxidation system is present in all examined tissues, however with differences in magnitude. More specifically expressed pathways are e.g. glyoxylate and D-amino acid degradation pathways. Auxiliary enzymes to the peroxisomal β-oxidation showed tissue specific expression, suggesting a high degree of tissue specific metabolite patterns, also being dependent on the metabolic state. The study also shows that PPARα is of major importance for the regulation in liver of the peroxisomal "transcriptome" during fasting.

Mitochondria degrade both fatty acids and amino acids and the mitochondrial acyl-CoA thioesterase 9 (ACOT9) was shown to hydrolyze both long chain acyl-CoAs as well as short chain acyl-CoA intermediates and products of branched-chain amino acid metabolism. Kinetic characterization of the enzyme suggests a thigh regulation of the activity during different metabolic conditions in the mitochondria.

Dietary ω-3 PUFAs from fish oil (FO) and krill oil (KO) cause different changes in lipid profiles and gene regulation when supplemented to mice. FO lowered most plasma lipids whereas KO only significantly lowered non-esterified fatty acids in plasma. FO showed a classical PPARα activation response by up regulating genes for fatty acid utilization and oxidation whereas KO down regulates genes for cholesterol and fatty acid synthesis.

LIST OF PUBLICATIONS

I. Reilly S.J.[*], **Tillander V**.[*], Ofman R., Alexson S.E.H. and Hunt M.C.
The nudix hydrolase 7 is an acyl-CoA diphosphatase involved in regulating peroxisomal coenzyme A homeostasis.
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J Biochem. 2008 Nov;144(5):655-63

II. **Tillander V.**, Lundåsen T., Svensson T., Hunt M.C. and Alexson S.E.H
Tissue expression and regulation of the 'Pexiome' in the mouse.
Manuscript

III. **Tillander V.**, Arvidsson Nordström E., Reilly J., Strozyk M., Van Veldhoven P.P, Hunt M.C., Alexson S.E.H
Acyl-CoA thioesterase 9 (ACOT9) in mouse may provide a novel link between fatty acid and amino acid metabolism in mitochondria.
Cell Mol Life Sci. 2013 Jul 18. Epub ahead of print

IV. **Tillander V**., Bjørndal B., Burri L., Bohov P., Skorve J., Berge R.K., and Alexson S.E.H.
Fish oil and krill oil supplementation differentially regulate lipid catabolic and synthetic pathways in mice.
Manuscript

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LIST OF ABBREVIATIONS

AA	Arachidonic acid
ABCD	ATP binding cassette transporter sub-family D
ACAA1	3-Oxoacyl-CoA thiolase 1
ACNAT	Acyl-CoA:amino acid *N*-acyltransferase
ACOT	Acyl-CoA thioesterase
ADP	Adenosine diphosphate
ALDH3A2	Aldehyde dehydrogenase 3A2
AMACR	Alpha-methylacyl-CoA racemase
ATP	Adenosine triphosphate
BAAT	Bile acid-CoA:amino acid *N*-acyltransferase
CACT	Carnitine-acylcarnitine translocase
COA	Coenzyme A
CPT1	Carnitine palmitoyltransferase 1
CPT2	Carnitine palmitoyltransferase 2
CRAT	Carnitine acetyltransferase
CROT	Carnitine octanoyltransferase
DBP	D-specific bifunctional protein
DECR	2,4-dienoyl-CoA reductase
DHA	Docosahexaenoic acid
DNA	Deoxyribonucleic acid
EPA	Eicosapentaenoic acid
ER	Endoplasmatic reticulum
FAS	Fatty acid synthase
FAD	Flavin adenine dinucleotide
FO	Fish oil
HACL1	2-hydroxyphytanoyl-CoA lyase
HF	High fat
KO	Krill oil
LCAD	Long chain acyl-CoA dehydrogenase
LBP	L-specific bifunctional protein
NADH	Nicotinamide adenine dinucleotide
NEFA	Nonesterified fatty acid
NR	Nuclear receptor
NUDT	Nudix hydrolase

PECI	Peroxisomal Δ^3-Δ^2-enoyl CoA isomerase
PL	Phospholipid
PPAR	Peroxisome proliferator-activated receptor
PUFA	Polyunsaturated fatty acid
RNA	Ribonucleic acid
SCP2	Sterol carrier 2 /3-oxoacyl-CoA thiolase
SREBP	Sterol regulatory element-binding protein
TAG	Triacylglyceride
VLACS	Very long chain acyl CoA synthetase
VLDL	Very low density lipoprotein

1 INTRODUCTION

1.1 LIPIDS

The word “fat” can mean many different things to people…
It can be the butter or the oil that you are using in your meal or it can be, the sometimes annoying, “soft” tissue on your belly.
Fat, in the sense above, is in fact a bit more complicated then just a “white/beige mass”. The butter and your fat pads do not only contain one type of fat molecule (or lipid), in fact they contain several types of molecules. In the case of our fat pads, they also contain various proteins that build up the fat storing cells or regulate if the lipids are stored or utilized as energy.

Lipids is a broad group of complex molecules that is composed of different fatty acids that are often esterified to glycerol or other alcohols (e.g. triacylglycerides and waxes), fatty acids that are esterified to glycerol/sphingosine (which are substituents of different phospholipids and sphingolipids), and sterols (e.g. cholesterol), lipid soluble vitamins, non-esterfied fatty acids and CoA-esters thereof.

This thesis will cover some aspects of lipids and fatty acids; how they get degraded and modified, how they can act as signaling molecules in gene transcription and cover new discoveries on some novel enzymes in fatty acid metabolism in both mitochondria and peroxisomes.

1.2 PEROXISOMAL AND MITOCHONDRIAL LIPID METABOLISM

The **mitochondrion** is quite often described as the “power plant” of the cell, due to its oxidative phosphorylation of ADP to generate ATP, which serves as the fuel for most reactions in the cell. The organelle harbors many different catabolic pathways such as for example degradation of amino acids and fatty acids that generate intermediates for the citric acid cycle (Krebs cycle), which further shuttles electrons and protons to the oxidative phosphorylation for ATP production.

However, mitochondria contribute in many other metabolic pathways in the cell, e.g. synthesis of heme and steroids, and also take part in other functions that are not directly connected to metabolism, like signaling in cell growth, and cell death by apoptosis. These functions will however not be further discussed in this thesis.

Most of the dietary lipids that we consume (of which approximately 90% are triacylglycerides, TAG) get degraded by the mitochondria if not in stored in lipid droplets within the cell until further use. However there are some lipids that first need

to be modified before being available as substrates for the mitochondrial fatty acid oxidation. For this action another organelle in the cell is essential, namely the peroxisome.

Peroxisomes belong to the family of "microbodies", which besides mammalian peroxisomes also contains yeast and plant peroxisomes, glyoxysomes (found in plant seeds) and glycosomes in unicellular eukaryotes such as *Trypanosoma* [1]. The organelle was actually first described morphologically by Johannes Rhodin in a PhD thesis from Karolinska Institutet and was later characterized as single membrane bound organelles containing different oxidase enzymes as well as the hydrogen peroxide degrading enzyme catalase by Christian De Duve and his colleagues. Due to these findings De Duve renamed the organelle as peroxisomes [2,3].
Peroxisomes are found in all cell types in the body except in erythrocytes and mature spermatocytes, and their widespread appearance in cells are today explained by their involvement in various essential metabolic pathways. The organelle contributes to glyoxylate metabolism, degradation of certain amino acids, purines, polyamines and certain long chain and complex fatty acids (see Peroxisomal lipid metabolism below). The organelle is also essential for synthesis of etherphospholipids and bile acids (for review see [4]).

Peroxisomes and mitochondria degrade fatty acids via so called β-oxidation. Common for the two β-oxidation systems is that the fatty acids need to enter the systems as acyl-CoA esters and that the fatty acids then undergoes four enzymatically catalyzed reaction steps: dehydrogenation (or oxidation in peroxisomes), hydration, a second dehydrogenation step and finally a thiolytic cleavage that in the end generates a 2-carbon shortened acyl-CoA and one acetyl-CoA (or in certain cases a propionyl-CoA) during each β-oxidation cycle. However, the reactions are carried out by different enzymes encoded by different genes and with different substrate preferences in the two organelles (for reviews, see [5-7])

1.2.1 MITOCHONDRIAL LIPID CATABOLISM

Acyl-CoAs will be β-oxidized to completion in the mitochondria, and the generated acetyl-CoA will be further degraded to CO_2 and H_2O in the Krebs cycle, or in certain cases be used for synthesis of other molecules. Both the β-oxidation process and the Krebs cycle will provide the oxidative phosphorylation with protons and electrons by the generation of NADH and the reduction of FAD to $FADH_2$ that in the end generates ATP from ADP in the electron transport chain.

Long chain fatty acids have to be transported into the organelle via the acyl-CoA/carnitine shuttle system that consist of the outer membrane located CPT1 (carnitine palmitoyltransferase 1), CACT (carnitine-acylcarnitine translocase) in the inner mitochondrial membrane and CPT2 (carnitine palmitoyltransferase 2) that is associated to the inner mitochondrial membrane facing the matrix. Long chain acyl-CoAs will be converted to long-chain acylcarnitine esters by CPT1 and shuttled to the

CACT that transport the carnitine esters across the inner membrane to CPT2, which in the end will reconvert the acylcarnitine esters to acyl-CoA esters that can then enter the β-oxidation system[8].

The mitochondrial β-oxidation machinery consists of proteins that are partly located in the mitochondrial matrix and partly associated with the inner membrane. The first dehydrogenation reaction is catalyzed by four acyl-CoA dehydrogenases with different substrate preferences. Short chain acyl-CoA dehydrogenase (SCAD), medium chain- (MCAD) and long chain acyl-CoA dehydrogenases (LCAD) are localized in the matrix, whereas the fourth enzyme, the very long chain acyl-CoA dehydrogenase (VLCAD coded by *Acadvl*) is membrane associated. VLCAD actually seems to be the major enzyme for handling long chain acyl-CoAs, at least in human (for review see [6]). The LCAD enzyme have a function during the degradation of methyl-branched and long-chain unsaturated fatty acids and seems to be of importance in murines as visualized by the severe phenotype in LCAD deficient mouse in which $C_{14:1}$ and $C_{14:2}$ carnitine esters and free fatty acids accumulate in plasma and bile [9-11].
The three following steps will be catalyzed by an enzyme complex called mitochondrial trifunctional protein (MTP or TFP, encoded by *Hadha* and *Hadhb*), at least for long chain *trans*-2-enoyl-CoAs. MTP is a hetero-octamer composed of 4 alpha-subunits and four beta-subunits of which the alpha-subunit harbors the enoyl-CoA hydratase and 3-hydroxyacyl-CoA dehydrogenase activities and the beta-subunit the 3-ketothiolase-activty. MTP prefers substrates of longer acyl chains with a preference for C_{16}-intermediates[12,13]. Medium - and short chain dehydrogenated intermediates will be further degraded by crotonase (enoyl-CoA hydratase), medium- and short chain hydroxyacyl-CoA dehydrogenases (M/SCHAD) and finally by a medium chain 3-ketoacyl-CoA thiolase (MCKAT)[14,15].

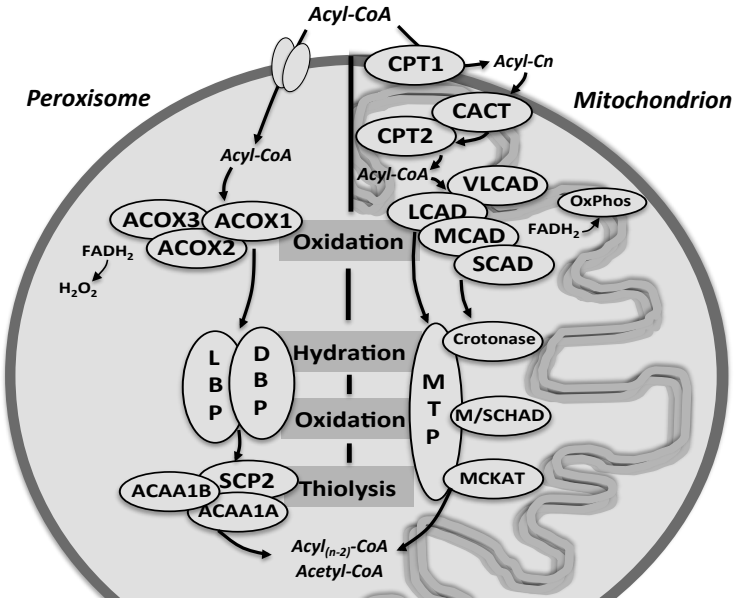
During the first dehydrogenation step in the β-oxidation of unsaturated fatty acids, either 2,4-dienoyl-CoAs from even-numbered double bond substrates such as $C_{18:1n-12}$, or 2,5-dienoyl-CoAs from odd-numbered double bond substrates such as oleic acid, $C_{18:1n-9}$, or from polyunsaturated fatty acids with double bonds at both odd and even-numbered positions, e.g. linoleic acid $C_{18:2\ n-9,12}$, will be generated. In both cases this will require an additional set of enzymes, namely 2,4-dienoyl-CoA reductase (DECR-1) and Δ^3,Δ^3-enoyl-CoA isomerase (ECI)[16,17] (and for review see [6]).

1.2.2 PEROXISOMAL LIPID METABOLISM

1.2.2.1 β-Oxidation

Peroxisomal β-oxidation is, like the mitochondrial version, a cyclic process that in the end generates a 2-carbon shorter acyl-CoA and one acetyl-CoA/propionyl-CoA in each cycle. However, the generated acetyl-CoA or $FADH_2$ or NADH can not be used in peroxisomes for ATP-production due to the obvious lack of oxidative phosphorylation that only occurs in the mitochondrion. The substrate preference for the peroxisomal β-oxidation is also different from mitochondria with a preference for long-chain and very long-chain fatty acids (that can also be unsaturated, methyl-branched or in their

dicarboxylic form), which are not (or only poor) substrates for the mitochondria. The peroxisomal system is active with medium -chain acyl-CoAs as well but do not seem to accept short chain metabolites [18-20]. Acyl-CoA esters that enters the peroxisomal β-oxidation only seems to undergo 2 to maximum 5 cycles of oxidation, which makes this fatty acid degradation a fatty acid chain-shortening system for the cell [21-24].



Schematic picture over the different enzymes involved in the different steps of peroxisomal (left hand side) and mitochondrial (right hand side) β-oxidation.

The first step is catalyzed by three (only two in human) FAD-containing acyl-CoA oxidases (ACOX1, ACOX2 and ACOX3) in mouse, but the electrons that are generated in this step are directly transferred to molecular oxygen, which in the end will generate H_2O_2. Together with several other peroxisomal oxidases, this is just one example of the reason why the hydrogen peroxidase degrading enzyme catalase is localized in peroxisomes, and inherited catalase deficiency may be classified as a syndrome [25,26].

The murine ACOX1 has preference for straight-chain acyl-CoAs, such as long chain saturated, unsaturated and dicarboxylic acyl-CoAs, and decreasing activity with increasing K_m with shorter substrates (e.g. C_4-C_6-CoA). ACOX2 is specific for the CoA-esters of the bile acid intermediates di- and trihydroxycoprostanoic acid that need one round of β-oxidation to be converted to the acyl-CoA esters of the mature bile acids, choloyl-CoA and chenodeoxycholoyl-CoA. ACOX3 is also called pristanoyl-CoA oxidase but also has some activity with straight chain unsaturated and dicarboxylic acyl-CoAs [27].

The two following steps are catalyzed by either of two unrelated multifunctional-proteins, D-specific bifunctional protein (DBP, MFE-2 or 17β-hydroxysteroid dehydrogenase type IV, encoded by *Hsd17b4*), or L-specific bifunctional protein (LBP, MFE-1, coded by *Ehhadh*). Both enzymes have a broad and overlapping substrate

specificity for the metabolites from all the oxidases. LBP, however seems to be indispensible for the degradation of dicarboxylic fatty acids to adipic and suberic acid, at least during fasting, as visualized in the mouse knock out model [28]. The DBP enzyme is on the other hand the most important enzyme of the two for the metabolism of bile-acid intermediates, methyl-branched chain fatty acids and very long-chain fatty acids based on the phenotype of the DBP-/- mice and the severity of the pathogenesis seen in patients with DBP-deficiency (for review see [29]). The last enzyme step in the cycle is the thiolytic cleavage, which in murine peroxisomes can be catalyzed by three different thiolases (but only 2 enzymes in human, ACAA1 and SCP2). 3-Oxoacyl-CoA thiolase A and B (coded by *Acaa1a* and *Acaa1b* in mouse and rat), prefer straight chain metabolites, and sterol carrier protein 2/3-oxoacyl-CoA thiolase (SCP2) acts on straight chain as well as methyl-branched chain 3-oxoacyl-CoAs. In addition, SCP2 contains a C-terminal domain that after proteolytic cleavage of the protein has a sterol carrier and lipid transfer function [30-32].

Like mitochondria, peroxisomes need an additional set of enzymes that can handle the β-oxidation intermediates of unsaturated fatty acids. The 2,4-dienoyl-CoA produced from double bonds at even positions will first be reduced by the peroxisomal 2,4-dienoyl-CoA reductase 2 (DECR-2), and then be further metabolized by Δ3,Δ2-enoyl-CoA isomerase (PECI) to 2-enoyl-CoA which then can re-enter the β-oxidation. Odd numbered unsaturated fatty acids can be degraded by two different pathways, one that only needs the action of PECI, or an additional route that requires $\Delta^{3,5}$-$\Delta^{2,4}$ dienoyl-CoA isomerase (ECH1) in combination with DECR2 and PECI. ECH1 enzyme shows dual localization to both peroxisome and mitochondria, which means that this extra degradation route also occurs in the mitochondria [33-35].

1.2.2.2 α-Oxidation

Diary products are the main source of the long-chain methyl-branched lipids, for example phytanic acid which is a degradation product from chlorophyll in ruminate animals. Phytanic acid is a 3-methyl branched fatty acid, which needs to be converted into a 2-methyl branched fatty acid (pristanic acid) for further degradation by the peroxisomal β-oxidation. To deal with this issue peroxisomes have a "one-carbon chain shortening system" called α-oxidation. Phytanic acid is probably esterified to CoA on the outside of the organelle, but can also be activated at the inside of the organelle by very-long chain acyl-CoA synthetase (VLACS). Phytanoyl-CoA is then hydroxylated to 2-hydroxyphytanoyl-CoA by phytanoyl-CoA hydroxylase (PHYH)[36,37]. The two following steps are the production of pristanal with the concurrent release of formyl-CoA by HACL1 (2-hydroxyphytanoyl-CoA lyase), and then the final conversion of pristanal to pristanic acid by a peroxisomal aldehyde dehydrogenase (FALDH-V, or ALDH3A2-v)[38-40]. Pristanoyl-CoA is chain-shortened three rounds by the peroxisomal β-oxidation into 4,8-dimethylnonanoyl-CoA that most likely is transferred to the mitochondria as a carnitine ester for further oxidation [41].

The β-oxidation of methyl-branched fatty acids is stereo-specific for *S*-isomers, however so is none of the α-oxidation enzymes so the α-oxidation pathway will

produce both the *R*- and *S*-isomer of pristanic acid. Localized to both organelles is a α-methylacyl-CoA racemase (AMACR) that converts the *R*-isomers of the methyl-branched fatty acid to *S*-isomers for the β-oxidation oxidation. AMACR is also essential for the oxidation of the bile acid intermediates that are produced as 25R-isomers and therefore needs to be converted into their 25S-steroeisomers before entering the peroxisomal β-oxidation [42].

1.3 AUXILLARY ENZYMES OF B-OXIDATION IN PEROXSISOMES AND MITOCHONDRIA

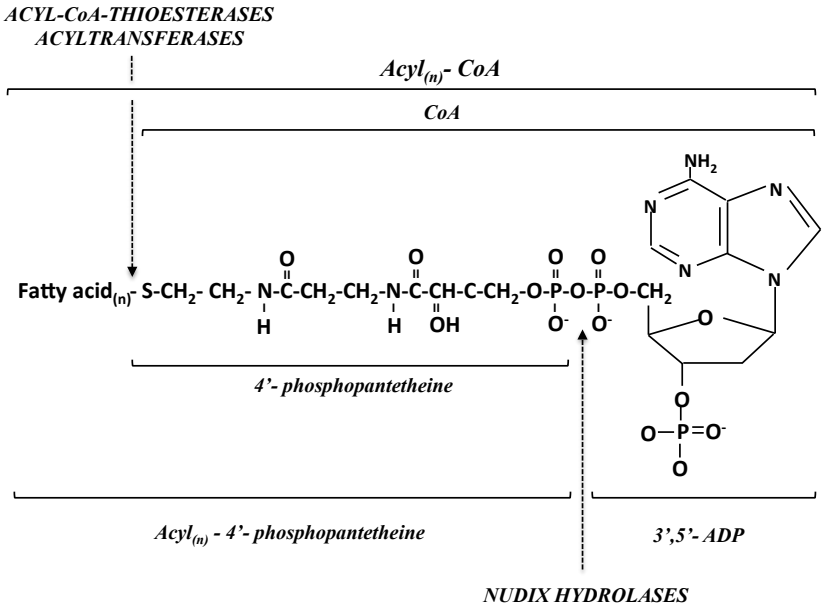
Transport of substrates and cofactors

Whereas the transport system of long chain fatty acids in to the mitochondria is quite well established (*see* Mitochondrial lipid metabolism section), the transport system in peroxisomes of these metabolites is still not yet clearly elucidated. However, it is generally believed that fatty acids enters the organelle in their acyl-CoA form due to the findings of so called ATP binding cassette (ABC) transporters in the membrane. These "half-transporters" form homo- or heterodimers to perform their ATP-dependent transport function, as judged from studies of the two ABC-transporters in *Saccharomyces cerevisiae* and studies on mammalian ABC-transporters using yeast 2-hybrid system [43,44]. Mammalian peroxisomes contain three of these ABC-transporters; ABCD1, also called X-ALD, due to the mutation of this protein that causes the neurological disease X-linked adrenoleukodystrophy, and ABCD2 (also known as adrenoleukodystrophy-related protein, ALDRP) and ABCD3 (also known as peroxisomal membrane protein 70 kDa, PMP70). Their substrate preferences have been proposed after findings of accumulations of metabolites in knockout mouse models, by overexpression in yeast PXA1/PXA2 (yeast peroxisomal homologs) mutants and by the accumulation of very-long chain fatty acids in X-linked adrenoleukodystrophy. Even if they have overlapping substrate specificities it seems that ABCD1 mainly transports very-long chain acyl-CoAs, also ABCD2 transports very-long chain acyl-CoAs but with a preference for more unsaturated species, and ABCD3 might be responsible for the transport of branched-chain acyl-CoA esters (for review see [45]). Recently, however, was the recombinant version of the *Arabidopsis thaliana* ABCD transporter Comatose shown to have thioesterase activity (*see section Acyl-CoA thioesterases*) during transport of acyl-CoAs into peroxisomes, and thereby releasing free fatty acids in the lumen, although it is not yet clear whether the hydrolysis occurs inside the peroxisome or on the cytosolic side of the membrane [46]. However, if mammalian peroxisomal ABC-transporters also have thioesterase activity similar to Comatose is not clear. Peroxisomes contain, as mentioned above, an acyl-CoA synthetase (VLACS) active on very long-chain and methyl-branched fatty acids that likely re-esterifies the transported fatty acids [36,47].

In the last step of the β-oxidation, the thiolase reaction, one extra CoA is needed for the production of the chain-shortened acyl-CoA ester with the concomitant release of acetyl/propionyl-CoA. Previously it was believed that the only known mechanism by

which CoA could enter the peroxisome was via these described ABC-transporters in the form of acyl-CoA esters. However, recently a re-characterization of the peroxisomal membrane protein 34 kDa (PMP34, also called SLC25A17 or solute carrier family 25 member 17) was accomplished, which showed that the before stated ATP-transporter activity of PMP34 is indeed a counter-exchange transporter with activity mainly with CoA, FAD and NAD^+, but also with products from peroxisomal metabolism, e.g. FMN, AMP and 3',5'-ADP [48].
No transporter of acyl-CoA out from the organelle is known (although PMP34 did show some low transport activity with acetyl-CoA), and due to the mass and bulkiness of the CoA moiety it is unlikely that chain-shortened acyl-CoAs is able to pass through the suggested peroxisomal membrane channels (made up by PXMP22) by diffusion since these channels only seem to allow passage of smaller solutes up to ≈ 350 Da [49].

The following section is an introduction to some auxiliary enzymes of lipid metabolism that by their action will regulate the amount of free CoA and acyl-CoA (and thereby also might affect the rate of β-oxidation) in these two organelles, and that will generate smaller metabolites able to leave the peroxisome by diffusion via peroxisomal membrane channels (and also the mitochondrion during certain situations) [50,51].



Structure of an acyl-CoA ester and the different cleavage sites of acyltransferases, acyl-CoA thioesterases and acyl-CoA active Nudix hydrolases, and the respective products thereof.

1.3.1 CARNITINE ACYLTRANSFERASES

Carnitine acetyl transferase (CRAT) and carnitine octanoyltransferase (CROT) are two acyl transferases that are active towards short chain CoA esters (mainly C_2- C_4-CoA) and straight as well as methyl-branched medium chain acyl-CoA esters respectively and produce the respective carnitine ester and free CoA [52-54]. The reversible reaction might also occur during high concentrations of acyl-carnitines and accessible amounts of CoA.

CRA/OT

Acyl-CoA + *L*-carnitine ➔ acylcarnitine + CoASH

CROT is an exclusive peroxisomal protein whereas CRAT is localized in both organelles due to two alternative translation start ATGs that generate either a 21-amino long N-terminal leader peptide, targeting this version to the mitochondria (and peroxisomes), or a shorter version lacking this leader peptide in which a version of the C-terminal peroxisomal targeting signal type 1 (PTS1) (-AKL in mouse) directs the protein to peroxisomes [55,56].

1.3.2 NUDIX HYDROLASES

Nudix- (nucleoside disphosphatase linked to another moiety X) hydrolase family is a huge group of enzymes that mainly consist of pyrophospohydrolases. Examples of substrates for this group are NTPs (nucleoside triphosphates), nuclear sugars and cofactors such as FAD or CoA and potential oxidized versions of the mentioned molecules. A commonly accepted function of this enzyme group has been stated to be a "housecleaning function" to eliminate potential toxic compounds or accumulating endogenous molecules in the cell (for review see [57]). Due to the focus of this thesis only CoA/acyl-CoA active enzymes and a NAD active peroxisomal enzyme will be further discussed due to their potential roles in regulating the availability of theses cofactors for the lipid catabolism in peroxisomes. Mammalian peroxisomes harbour three Nudix hydrolases, one active towards nicotinamide nucleotides and two that are active with CoA and CoA derivatives.
Human NUDT12 was cloned and characterized in 2003, and was found to be active with nicotinamide nucleotides, preferably towards their reduced form; NADPH and NADH to produce NMNH and 2',5'-ADP and AMP respectively, but also showed some activity towards e.g. FAD, NAD^+, $NADP^+$ [58]. Due to its activity the enzyme contributes to the regulation of the NAD+/NADH pool in peroxisomes and thereby also potentially influence the rate of β-oxidation since the dehydrogenase step performed by the two multifunctional proteins needs NAD^+.

Two Nudix hydrolases, NUDT19 and NUDT7α, have been shown to be active on CoA and acyl-CoA esters of different chain-lengths in peroxisomes. Both are active on the CoA-moiety cleaving off 3',5'-ADP from the molecule leaving a 4'-phosphopantetheine (in the case of CoASH) or 4'-acylphosphopantetheine in the case of acyl-CoAs as a substrate.

NUDT

Acyl-CoA ➔ 4'-acylphosphopantetheine + 3',5'ADP

NUDT19 was identified in 2006 (at that time known as RP2p) as an abundant peroxisomal protein in a proteomic analysis of mouse kidney peroxisomes. The protein was cloned and expressed and was shown to be active with CoA as well as with a wide range of acyl-CoA esters [59]. NUDT7α was first stated to be a nudix hydrolase "specific" for CoA, oxidised CoA, and short-chain derivatives thereof [60]. However our group showed that the enzyme in fact prefer medium chain acyl-CoA esters (*Paper I*) [61]. Although these two enzymes seem to have overlapping substrate preferences, there is a major difference in their actual activity towards the same substrates as judged from published kinetic data, whith NUDT7α being the most active enzyme of the two with these CoA-esters. The re-characterization of NUDT7α will be further discussed in the Results section.

The mitochondra might harbour one putative CoA-active enzyme, NUDT8, and a predicted NADH diphosphatese, NUDT13, however no published characterization of these proteins is available (unpublished work by S. Abdel Raheim and McLennan)[57,58].

1.3.3 ACYL-COA THIOESTERASES

Acyl-CoA thioesterase activity is ubiquitously expressed and the enzymes responsible for the activity, acyl-CoA thioesterases (ACOTs) are found in several cellular compartments. These enzymes hydrolyze the thioester bound of acyl-CoA esters and thereby release free coenzyme A and the corresponding free fatty acids (for review, see [62]).

ACOT

Acyl-CoA ➔ free fatty acid + CoASH

These enzymes have previously been characterized also as "acyl-CoA hydrolases" and "deacylases" and are found in many organisms from prokaryotes (e.g. bacteria, like *Eschericha coli* to eukaryotes (from yeast to human). The wide expression among species, tissues and subcellular compartments suggest that they have important roles in cellular metabolism although the exact physiological functions of these enzymes are

still not well established, in spite of more and more studies detailing their structures, regulation and in certain cases what happens if they are silenced/deleted (for reviews see [62,63]). However, based on the enzymatic reaction it is easy to envisage a function of these enzymes to regulate the levels of different acyl-CoAs, free fatty acids and free CoASH in the different cellular compartments. From 1952, when the first acyl-CoA thioesterase activity was described and the responsible enzyme was partly characterized [64], a number of ACOTs with diverse substrate specificities have been identified and characterized. Their combined activity covers in principal the whole range of acyl-CoAs with activities on short- to long straight-chain-acyl CoAs, saturated as well as unsaturated fatty acids, to methyl-branched and dicarboxcylic acyl-CoA esters.
A revised nomenclature was published by Hunt et al. in 2005, establishing the family root symbol for the enzymes to be ACOT [65]. At that time ACOT1-12 were known and since then the enzyme group has expanded further to now (2013) contain 15 and 12 ACOTs in mouse and human respectively (for rev see [63]). ACOT11 is also known as THEM1, ACOT13 as THEM2, and THEM4 and 5 may tentatively be included in the thioesterase family as ACOT14 and 15 respectively. The ACOT enzymes can be further divided into two different classes depending on their structure; *Type-I acyl-CoA thioesterases* that belong to the α/β-hydrolase superfamily of proteins and *Type-II acyl-CoA thioesterases* that belong to the "hot dog" fold thioesterase family.

1.3.3.1 Type-I ACOTs

This group consists of ACOT1-6 in mouse and ACOT1-4 and ACOT6 in human, and they all share a high sequence and structural homology to each other. In fact all genes coding for these proteins are located in a gene clusters on chromosome 12D3 in the mouse and on chromosome 14q24.3 in human [66]. All genes consist of 3 exons that code for proteins with a N-terminal domain (except for human ACOT6), which might have a role in the substrate preference or the regulation of the enzyme, and a C-terminal α/β-hydrolase catalytic domain. The α/β-hydrolase domain contains a conserved catalytic triad of a serine (which acts as the nucleophile and is located in the so-called nucleophilic elbow), an aspartic acid and a histidine, which constitute the active site that seems to be located in between the two domains (at least in the crystal structure of the human ACOT2) [67,68]. In spite of their high sequence similarity, they have different substrate preferences (but sometimes overlapping activities), distinct tissue expression and to localize to different cellular compartments. Common for them all is however their strong induction in expression in response to PPARα activation, at least in the mouse [69].

1.3.3.2 Type-II ACOTs

This group consists of ACOT7-15, and in contrast to the type-I ACOTs these enzymes only share low degree of sequence similarity and the corresponding genes are spread out over the genome. However, they are structurally related due to their common "HotDog fold structural motif". This structural motif is composed of a 5-7 stranded antiparallel β-sheet that build up a bowl-shaped "bun" in which a α-helix rest, similar to a sausage, hence the HotDog fold (for reviews see [67] and [63,70]).

Most of the enzymes in this group (ACOT7-12) consist of so-called tandem hotdog fold domains (with two "active" sites), which dimerize to form one active site (based on the structure of ACOT7). These dimers can then oligomerize to form e.g. trimers of dimers (see the structure of ACOT7)[67,71]. In contrast to ACOT7-12, ACOT13-15 are single HotDog fold proteins.

1.3.3.3 ACOTs, their cellular localization and activity

Peroxisomal ACOTs

Out of the mentioned type I ACOTs, murine ACOT3-6 are peroxisomal proteins localised to the organelle by variants of the C-terminal PTS1. ACOT3 is active towards medium-chain (from C_6 -CoA) to long-chain (up to C_{26}-CoA) straight chain acyl-CoAs, with a preference for C_{14}-C_{16}-CoA. ACOT5 is similar to ACOT3 active towards straight chain acyl-CoAs, but with a preference for medium chain acyl-CoAs [72]. Mouse ACOT4 and ACOT6 have more specific activities for certain substrates with ACOT4 being only active towards the short-chain dicarboxylic acyl-CoAs succinyl-CoA and glutaryl-CoA, and ACOT6 with the long chain methyl-branched acyl CoA esters of phytanic- and pristanic acid [73,74]. ACOT3 and ACOT5 are only found in rodents and human thus only contain one type I peroxisomal thioesterase, ACOT4. Interestingly, human ACOT4 is active against all the substrates that were unique for the murine ACOT3, ACOT4 and ACOT5 [66]. Therefore it is likely that rodents evolved more specialized enzymes via a series of gene duplications. In human the first exon in the ACOT6 gene is not transcribed, resulting in a protein that lacks the N-terminal domain and ends in –SKI, which did not target the protein to peroxisomes in human skin fibroblasts [66].

ACOT8 was shown to be a thioesterase with very broad substrate specificity, in fact hydrolyzing all tested CoA-esters including short-chain to long chain acyl-CoA esters as well as methyl branched acyl-CoA esters and the CoA-esters of the bile acid intermediates. However, unlike the type I ACOTs this enzyme is inhibited by CoA with an IC_{50} of 10-15 μM suggesting a role in sensing intraperoxisomal acyl-CoA/CoA levels [75]. A second type II thioesterase, ACOT12, has been shown to have a dual localization in peroxisomes and the cytosol of rat liver, however only rodent ACOT12 contain a variant of the PTS 1 [76]. ACOT12 is a short chain ACOT active mostly on acetyl-, propionyl- and butyryl-CoA[77,78].

Mitochondrial ACOTs

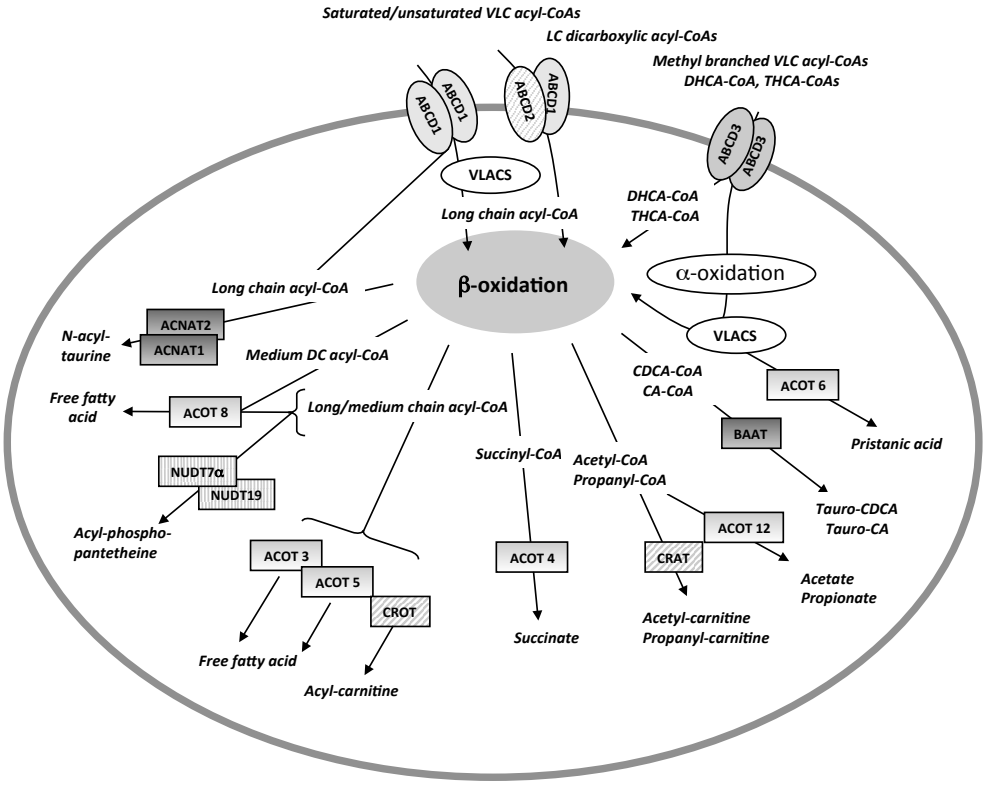
ACOT2 is the only type I thioesterase with potential mitochondrial localizations. The enzyme is a long-chain thioesterase with highest activity with C_{14} to C_{18}-CoA, and cloning of the gene coding for ACOT2 revealed a N-terminal mitochondrial-targeting signal for the enzyme [69,79]. During the initial characterization of mitochondrial long chain thioesterase activities, prior to the molecular cloning era, were in fact two enzymes found in rat liver mitochondria. In addition to ACOT2 (at that time called MTE-I) also an enzyme of higher molecular mass, called MTE-II, was detected in rat liver mitochondria and shown to be active against long-chain acyl-CoA esters but with a much wider chain length acceptance (from C_8 to C_{20}) [79]. MTE-II was later identified

as an alternative splice variant of ACOT7 (*Acot7_v2)* of which many other splice forms exist that are cytosolic [80].
ACOT9 and ACOT10 were first described as MT-ACT48, and characterized as long chain acyl-CoA thioesterases due to the activity on C_{14}-CoA. However, *Acot10* is a (functional) retrogene found only in mouse and is unlikely to be expressed at appreciable amounts. In an extended characterization of ACOT9 we showed the real substrate preference for ACOT9 to be much broader than previously suggested, which will further be discussed in *paper III* [81,82].
ACOT11 (or Them1) is also known as BFIT (brown fat inducible thioesterase) due to its high abundance in brown adipose tissue and its strong induction in expression during cold exposure in mice [83]. Ablation of Acot11 showed, somewhat unexpectedly, that the Them1-/- mice are protected against diet-induced obesity, hepatic steatosis and WAT inflammation [84]. In addition these mice showed increased energy expenditure indicating a function for ACOT11 in preserving energy. The cellular localization of ACOT11 is not fully clear, the protein was found in the cytosol, ER and in mitochondria in mice, whereas one of two ACOT11 splice variants showed mitochondrial localization in human [84,85]. ACOT11 seems to be a long chain acyl-CoA thioesterase active against $C_{12-}C_{16}$–CoA [83,85].
During the characterization of human Them2 (ACOT13), activity was detected with polar aromatic acyl-CoA esters (e.g. phenylacetyl-CoA, like for its possible ortholog in *E. coli*). However, the phenylacetic acid degradation pathway probably does not exist in human, while its activity against medium- to long-chain acyl-CoAs was later suggested to be the most important physiological substrates for the enzyme [86,87]. The enzyme was found to be associated with the mitochondria but also in the cytosol and to interact with PC-TP (phosphatidylcholine transfer protein), which increases the activity of Them2 (for review see [88]). The *Them2-/-* mouse are also resistant to hepatic steatosis and enhanced glucose production in liver during high fat feeding, and was proposed to have an important role to regulate hepatic glucose and lipid metabolism by regulating the levels of ligands for lipid binding NR such as PPARα [89]. The sixth ACOT with possible mitochondrial localization is Them4 (or ACOT14), which was also shown to be active with medium- to long-chain acyl-CoAs [90], and the last (known today) mitochondrially localized thioesterase, ACOT15/Them 5, which is also active with long-chain acyl-CoAs, both saturated as well as unsaturated, with highest activity with palmitoyl-CoA ($C_{16:0}$–CoA). Them5 was shown to have a critical role in cardiolipin remodeling and *Them5-/-* mice show deregulated lipid metabolism, development of fatty liver and effects on mitochondrial function [91]. Taken together mitochondria contain several long-chain ACOTs with crucial functions that are tightly linked to regulation of lipid metabolism.

1.3.4 *N*-ACYLTRANSFERASES

Related to the mentioned Type I thioesterases is a small group of three acyl-CoA:amino acid *N*-acyltransferases. These three genes are located in a small cluster on chromosome 4B3 in the mouse of which one gene codes for the bile acid-CoA:amino acid *N*- acyltransferase (BAAT) and the two other genes code for one previously

characterized acyl-CoA:amino acid *N*-acyltransferase (ACNAT1), and one predicted, still uncharacterized, acyl-CoA:amino acid *N*-transferase (ACNAT2**)**. BAAT is responsible for the conjugation of the chain shortened bile acids choloyl-CoA and chenodeoxycholoyl-CoA to taurine (in murine) or taurine or glycine (in humans). BAAT is found in many species, however it is not yet fully established whether the protein is entirely peroxisomal, or if it has dual localization in peroxisomes and cytosol, which might depend on cell type and species [92-94]. ACNAT1 has been shown to conjugate long chain-saturated acyl-CoA to taurine [95]. The protein coded by *Acnat2* show 92% amino acid sequence identity to ACNAT1, and was predicted to perform a similar activity as ACNAT1, however attempts to express the protein has not yet been successful [96].



Schematic picture of the auxiliary enzymes of peroxisomal lipid metabolism, their substrates and products (in mouse).

1.4 LIPID ACTIVATED RECEPTORS AND NUCLEAR TRANSCRIPTION FACTORS

1.4.1 PPARs - PEROXISOME PROLIFERATOR-ACTIVATED RECEPTORs

Peroxisome proliferator activated receptors is a group of ligand activated transcription factors that belong to the subgroup of nuclear hormone receptors. These nuclear receptors form heterodimers with the RXR (9-*cis*-retinoic acid receptor) in the nucleus, and during ligand activation they regulate genes involved in lipid- and glucose metabolism (both catabolic and anabolic) as well as genes for differentiation, immune

response and cell growth. These receptors were named based on the finding that the first identified receptor, PPARα, is activated by a group of chemicals (e.g. clofibrate) that cause peroxisomal proliferation in rodents. A number of other substances were later on shown to mediate a similar effect, and the group of peroxisomal proliferators (PPs) include today a vide range of hypolipidemic drugs (like different fibrates and Wy-14,463) and some synthetic xenobiotics (e.g. industrial phthalate monoester plastisizers), that during prolonged administration in high doses caused liver carcinomas in mice an rats. These PP activated nuclear transcription factors were later found out to be a family of three isoforms [97]. These three isoforms (PPARα, -δ and -γ) are today known to differ in their ligand preference, specificity of target genes and in their tissue expression. The major focus in this thesis will be on the PPARα isoform. Ligands to the different PPARs were later shown to be not only exogenous chemical compounds and pharmacological substances but also dietary fatty acids as well as endogenously synthesized fatty acids and derivatives thereof (e.g. phospholipids, eicosanoids and endocannabinoids).

1.4.1.1 PPARα

PPARα was the first isoform to be cloned and was characterized as the transcription factor to be responsible for most of the actions of PPs seen in murine livers [98]. The activation of PPARα increases the levels of enzymes in mitochondrial and peroxisomal (straight chain) β-oxidation and the ER located enzymes of fatty acid ω-oxidation (e.g. CYP4A10). It also regulates the transcription of genes in lipid/fatty acid transport in cellular membranes and for trafficking within the cell, and is essential during the adaptation to fasting by increasing the expression of genes needed for ketogenesis and gluconeogenesis. This is reflected in the PPARα-/- mouse who suffer from severe hypoketonemia, hypoglycaemia, elevated levels in plasma of free fatty acids and accumulation of liver lipids during prolonged fasting [99,100]. The receptor is most highly expressed in tissues known for high lipid metabolism such as brown adipose tissue, liver, kidney, heart, skeletal muscle and also in the intestinal epithelium. As mentioned above, the receptor is strongly activated by fibrates but is also activated by a variety of fatty acids. PPARα preferably bind to long-chain (poly)unsaturated fatty acids, very-long chain fatty acids, long chain methyl branched fatty acids, eicosanoids and sulphur-substituted fatty acids and according to Hostetler et al. with a preference for their respective CoA esters [101-103].
In 2009 was also the endogenously synthesized and FAS (fatty acid synthase) dependent phospholipid 1-palmitoyl-2-oleoyl-*sn*-2-glycerol-3-phophocholine (16:0/18:1-GPC) shown to be a ligand for the PPARα, however with a lower affinity than for Wy14,643. Also exogenous supply of 16:0/18:1-GPC to cultured cells or infusion directly into the portal vein of PPARα+/+ mice was shown to increase the expression of *Acox1* and *Cpt1a*, demonstrating that this compound does not necessary have to be endogenously synthesized for the effect [104]. In fact, FAS was recently shown to localize both to the ER and cytosol where the cytosolic form showed a phosphorylation dependent activation of PPARα during fasting and when insulin signaling is low (and therefore the phosphorylation by mTORC1) [105].

1.4.1.2 PPARγ

PPARγ, present in two splice variants, is mostly expressed in adipose tissue and in different immune cells and mediates more of an anabolic effect then the other two PPARs by its regulation of genes that promote lipid storage by enhancing FA transport, TAG synthesis and lipid droplet formation. PPARγ is in fact essential for normal embryo development and differentiation of adipocytes demonstrated by the severe phenotype of the PPARγ-/- mouse model [106]. PPARγ activation by thiazolidinedione's (such as rosiglitazone and pioglitazone) improves insulin sensitivity and lower blood glucose levels in type 2 diabetes patients, however prolonged treatment also show side effect coupled to glucose and lipid metabolism in both muscle and liver tissues as well as weight gain and osteoporosis (for rev see [107]). Some suggested endogenous ligands to the PPARγ is the eicosanoid 15-deoxy $\Delta^{12,14}$ – prostaglandin J_2 and oxidized metabolites of linoleic acid such as 9- and 13-hydroxy octadecadienoic acid (9-HODE and 13-HODE) which are common components of oxidized LDL (for review see [108]).

1.4.1.3 PPARδ

PPARδ is widely expressed throughout the body and is the most dominating PPAR isoform in skeletal muscle. PPARδ seem to have more complex actions with similar gene regulatory actions as PPARα by promoting lipid catabolism, and PPARγ like effects on glucose metabolism in muscle (for review see [109]). Also its ligand preference seem to be quite similar to PPARα in that it is activated by unsaturated fatty acids and eicosanoids, however not with hypolipidemic agents such as clofibrate [103].

1.4.2 OTHER LIPID ASSOCIATED TRANSCRIPTION FACTORS

HNF4α (hepatocyte nuclear factor 4α) was in 1998 claimed to bind saturated acyl-CoA esters, however it is still under debate whether CoA-esters are ligands for the receptor due to the finding of a to narrow ligand binding pocket for the protein to allow CoA-ester bindings, and the observations from crystal structures of the protein in complex with different fatty acids [110] [111]. However, findings of thioesterase activity by the nuclear receptor in complex with fatty acids bound in the binding pocket may explain both findings of acyl-CoA and fatty acid binding in the protein [112].
SREBPs (sterol regulatory element binding proteins) are found in two isoforms, SREBP-1 (a and c, where c is the dominating form in liver) that regulate the expression of lipogenic genes, such as fatty acid synthase (FAS) and acetyl-CoA carboxylase 1a, and SREBP2 that regulate genes involved in the cholesterol synthesis and transport. Fatty acids (or derivatives thereof) cannot bind as ligands to these proteins, but at least polyunsaturated fatty acids (PUFAs) have been shown to down regulate the expression of SREBP1-c. The transcriptional down regulation of SREBP1-c has been suggested to be a caused of inhibition of LXRα (liver X receptor α, a oxysterol activated transcription factor) that, among other genes, control the expression of SREBP1-c. However PUFAs have been shown to inhibit the proteolytic cleavage of the precursor protein of SREBP in the ER, which is required for the mature protein to go to the

nucleus and perform its gene regulatory events, and that this also exerts a negative feed-back regulation on its own expression [113].

1.5 Ω-3 FATTY ACIDS

Since the 1970s when the low incidence of cardiovascular diseases in Greenland Inuit were pointed out by Dyerberg et al. has consumption of fish and preferably fatty fish been associated with several health promoting effects, especially on cardiovascular health [114,115]. The Swedish National Food Agency recommend that we eat fish (and sea food) 2-3 times per week because of its high content of certain vitamins (e.g. vitamin D) and minerals (e.g. iodine and selenium) and due to its high content of long chain ω-3 polyunsaturated fatty acids (ω-3 PUFAs). Nordic Nutrition Recommendation for intake of *cis*-PUFAs is between 5-10 E%, including about 1 E% as ω-3 fatty acids [116]. However, due to generally low intake of fatty fish, fish oil is commonly consumed as a nutritional complement. Recently krill oil has become a significant product on the market as a complement to fish oil.

The ω-3 PUFAs EPA (eicosapentaenoic acid, $C_{20:5n-3}$) and DHA (docosahexaenoic acid, $C_{22:6n-3}$) are mainly found in fish, shellfish, seaweed and other marine derived foods and, of course, in fish oil. Today they are extensively studied and established as bioactive molecules that affects both lipid and glucose metabolism by acting as ligands for different nuclear transcription factors that also have impact on the immune system by causing changes in an array of different lipid signaling molecules in the system. Incorporation of these fatty acids into cellular membranes makes them available as substrates for phospholipases to release them for access to prostaglandin (PG), thromboxane (TX) and leukotriene (LT) synthesizing enzymes and therefore compete with their reactions towards arachidonic acid. These ω-3 "versions" of PGs, TXs and LTs have shown to cause less inflammatory responce in comparison to the PGs, TXs and LTs generated from arachidonic acid (for review see [117]). Recently has also the resolution of acute inflammation been shown to be a lipid mediated process in which so called resolvins produced from EPA and DHA, and protectins and maresins from DHA accelerate the resolution of inflammation (for review see [118]).

Increased plasma triacylglycerol (plasma TAG) levels is a cardiovascular risk factor and fish oil supplementation (at least at high "pharmaceutical doses" ≈ 3 g or more ω-3 PUFAs per day) has been shown to decrease elevated plasma TAG. Many of these effects of ω-3 PUFAs have been ascribed to the activation of PPARα in the liver in a fibrate-like manner by increasing the plasma VLDL-clearance, decrease the VLDL-TG production by the liver and to turn on the fatty acid degradation systems in hepatocytes [119,120]. However, PUFAs are as described above ligands to several nuclear receptors, which is also true for the ω-3 class of PUFAs that are known to both affect SREBP1c negatively and to bind and activate all different PPARs. So the systemic effects of increasing the levels of ω-3 PUFAs can also be due to a higher utilization of fatty acids in muscle (due to activation of PPARδ), a decreased release of NEFA and/or

increased uptake of plasma TAG by adipose tissue due to activation of PPARγ and decreased *de novo* lipid synthesis by inhibiting SREBP1c signaling[121].

2 AIM

The aim of these studies was to investigate expression and activity of novel enzymes related to peroxisomal and mitochondrial lipid metabolism by:

In depth characterization of NUDT7α and ACOT9, two novel enzymes in peroxisomal and mitochondrial lipid metabolism, respectively.

Investigation of the transcriptome of the peroxisome ("Pexiome") to identify tissue expression patterns of metabolic pathways, to identify novel associations of genes to established metabolic pathways from co-expression of peroxisomal protein coding transcripts, and to explore expression of the so-called auxiliary enzymes of peroxisomal β-oxidation, and also to investigate the regulation of the Pexiome in response to exogenous PPARα stimulation with PPs and endogenous PPARα stimulation during fasting.

Investigation on how these two organelles contribute to lipid metabolism during treatment with different biologically active lipids in the form of fish oil and krill oil.

3 METHODS

3.1 RECOMBINANT PROTEIN EXPRESSION

In *Paper I* and *III* the open reading frames of *Nudt7α* and *Acot9,* respectively, were amplified from mouse kidney total RNA, using primers with *Nde1* and *Xba1* sites. Products were then cloned into the *Nde1* site in the pET-16b vector (yielding a His-tagged fusion protein), or the *Xba1* site in the pMal-C2X vector (yielding a maltose-binding fusion protein), respectively. The different plasmids were then used to transform BL21(DE3) pLysS cells, which were cultured in Luria-Bertani medium with appropriate antibiotics, and in the case for ACOT9 with additional glucose. Protein expression was induced in the bacteria by 1 or 0.5 mM isopropyl-1-thio-β-D-galactopyranoside, after reaching an optical density (OD_{600}) ≈ 0.6. After 3 h the bacteria were harvested by centrifugation and the pellets were frozen until protein extraction. Bacterial pellets were resuspended in BugBuster (with Benzonase) and incubated ≈ 15 min before centrifugation. Supernatants were then used for protein purification using either a His-Trap™ column or amylose resin respectively. Elution of respective protein was done with increasing concentration of imidazole in the case for NUDT7α and maltose for ACOT9. Protein concentrations were measured using the Bradford method.

3.2 ENZYME ACTIVITY MEASURMENTS

The assay for measuring the activity of NUDT7α in *Paper I* is a coupled spectrophotometric assay in which the total released phosphate in the incubation was measured after reaction with calf intestine alkaline phosphatase and further reaction with ascorbic acid ammonium molybdate in H_2SO_4. Absorbance of samples (containing NUDT7α and substrate) and controls (only substrate) were measured at 820 nm and the amount of product was calculated based on A_{820}=0.260 for 10 nmol inorganic phosphate (P*i*).

The thioesterase activity assay used in *Paper III* is a 5,5'-dithiobis(2-nitrobenzoic acid) (DTNB) dependent analysis in which the released CoA reacts with DTNB to form 5-thio-2-nitrobenzonate (with a molar absorption coefficient of 13,600 M^{-1} x cm^{-1}), which is measured at 412nm. Activity was calculated for the linear rate of activity. Hydrolysis of the thioester bound was measured in PBS at 232 nm for the CoASH inhibition measurements with ACOT9.

In *paper I* was a HPLC based method used to quantitate formed 3',5'-ADP in isolated peroxisomes and in *paper III* to quantitate released CoA and visualize the loss of acyl-CoAs in a substrate mixture with time. Reactions were in both cases stopped by acidification and the products were separated on a C18 column by elution with a gradient of increasing ratio of acetonitrile to ammonium phosphate buffer (up to 1:1,

and thereafter isocratic flow). The appearance of the nucleotide was measured by an UV-detector at 260nm.

3.3 LIPID ANALYSIS IN PLASMA AND IN LIVER

In *paper IV* different lipid analyses were done on liver and plasma. Lipoproteins were analyzed by size-exclusion chromatography on plasma samples from individual mice. Total lipids from plasma and liver were extracted using $CHCl_3$:MeOH in a 1:2 (v/v) ratio according to Bligh and Dyer, evaporated by nitrogen flow and re-dissolved in isopropanol before further analysis. Lipid extracts were then analyzed spectrophotometrically by using different enzymatic assays (kits for total TAG, cholesterol and PL) in a Hitachi 917 system. Lipid extracts were further used for gas chromatography (GC) analysis of the fatty acid composition of plasma and liver. Lipids were first separated using thin layer chromatography on silica plates developed with hexane:diethylether (1:1) and TAG and PL were collected for further GC-analysis.

3.4 GENE EXPRESSION

Gene expression analysis was performed in *all papers* in principal as described here. Total tissue RNA from individual samples was extracted after tissue homogenization using the MagMax system (Applied Biosystems) including DNase treatment except that in *paper I* total RNA was extracted using the Trizol reagent protocol. Total RNA concentration was measured spectrophotometrically using NanoDrop (NanoDrop Products). RNA quality was determined using the Experion automated electrophoresis system (Bio-Rad) except for *paper I* where agarose gels with ethidium bromide staining was used for total RNA quality determination. Relative gene expression was measured by Real-Time PCR either using Taqman gene expression assays (Applied Biosystems) or SYBRgreen primers for different genes. The $2^{\wedge(-\Delta\Delta Ct)}$ method was used to calculate relative expression, mostly using *18S* as the reference gene. The real-time measurements with the Taqman low-density arrays were performed at the core facility BEA (Bioinformatics and Expression Analysis) at Karolinska Institutet/University Hospital.

3.5 CLUSTERING ANALYSIS

In *Paper II* two different clustering analyses were performed on gene expression data. Hierarchical clustering was performed to investigate similarity between tissues in their expression of the investigated gene set. Complete-linkage ("diameter" or "maximum" linkage method) was used here, which take into account the greatest difference between two groups and join each group with the group that have the shortest distance between each other for each round of clustering.
K-means is a partitioning method that divides samples into a pre-decided set of clusters *(k)*. The initial means for cluster partitioning is randomly chosen and is updated iteratively until convergence for the mean (centroid) is reached or the maximum number of iteration steps is reached. The aim of this clustering process is to allocate all samples into clusters so that the mean values (distance between samples, in *Paper II* the

gene pattern in the tissues) in each cluster will be as small as possible. Since the final cluster number needs to be decided beforehand, a number of k-means clustering attempts was done, in this case k=5-9, based on the assumption that too many final groups would not be preferable due to the increasing number of single-gene clusters. Cluster evaluation was then performed to see what value of k would yield the best result. For this, the Dunn index was used that takes into account how compact the clusters are and how well they are separated.

4 RESULTS AND DISCUSSION

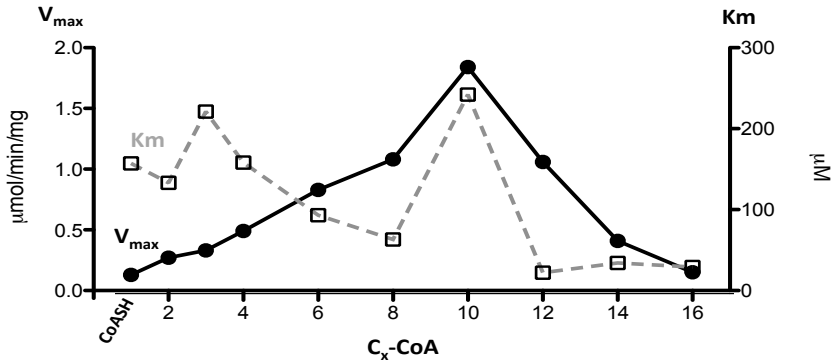
4.1 PAPER I

"The Nudix Hydrolase 7 is an Acyl-CoA Diphosphatase Involved in Regulating Peroxisomal Coenzyme A Homeostasis"

In 2000 the *Pcd1* gene in *S. cerevisiae* was shown to encode a peroxisomal nudix hydrolase that was found to hydrolyze coenzyme A, but with a preference for oxidized CoA (CoASSCoA) and some other CoA-derivatives and therefore stated to have a detoxification role in the organelle [122]. Gasmi et al. cloned and expressed the mouse homologue to this enzyme in 2001 and NUDT7 was established as a peroxisomal CoASH diphosphatase [60]. However, the activity of the enzyme was only tested with a limiting number of substrates according to the results in that publication. In 2006 was another Nudix hydrolase (NUDT19) characterized from mouse kidney and shown to be active against the CoA-moiety of longer acyl-CoA esters [59]. Due to these findings we decided to reinvestigate the activity of NUDT7.

Two isoforms of NUDT7 had previously been described, *Nudt7α* and *Nudt7β*, of which the β-isoform is inactive due to a loss of 20-amino acid that destroys the nudix hydrolase motif [60]. In this study we identified a third isoform, *Nudt7γ*, however due to the findings of very few expressed sequence tags (ESTs) in databases corresponding to the γ-variant and that the mRNA expression of the transcript was approximately 20 times lower then *Nudt7α*, this isoform was not further investigated in this study even if it would code for an active nudix hydrolase.

Nudt7α was expressed in *E. coli* as recombinant protein and the activity with different acyl-CoAs was scanned at a fixed concentration of 200 μM which revealed that NUDT7*α* in fact was active with CoASH, as expected, but also towards a wide range of different acyl-CoA esters including the bile acid precursors choloyl-CoA and trihydroxycoprostanoyl-CoA, as well as to some unsaturated acyl-CoA esters. Kinetic parameters were investigated for CoASH and the straight chain saturated acyl-CoA esters and showed that NUDT7α was most active against substrates ranging from C_6- to C_{12}-CoA, and based on calculation of k_{cat}/K_m lauroyl-CoA was the best substrate.

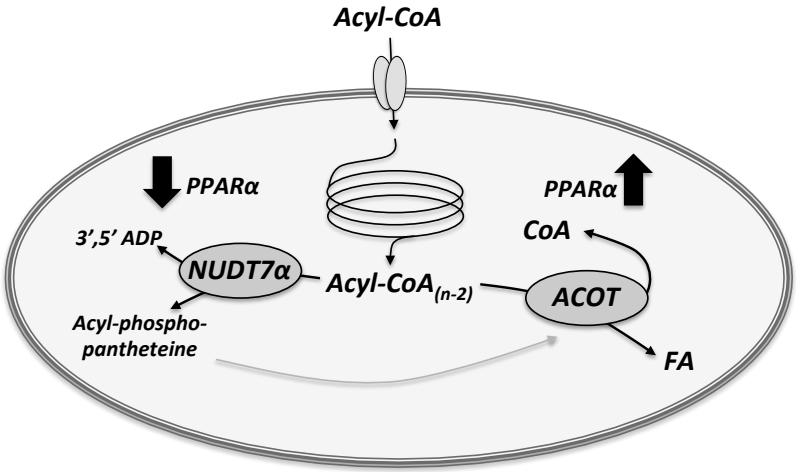


Graph presenting mean V_{max} (black circles) and K_m (open squares) of recombinant NUDT7α.

The relative expression of the mRNA transcript that codes for *Nudt7α* was investigated by real-time PCR and showed highest expression in liver, BAT, heart and WAT, and low expression in e.g. kidney, lung and brain.
Liver, BAT, heart and WAT are all tissues that contain high amounts of peroxisomal β-oxidation enzymes and due to this apparent tissue co-expression pattern we hypothesize that NUDT7 has a regulatory function in the degradation of fatty acids in peroxisomes. Although kidney is also a tissue that contains high amounts of peroxisomes as well as high transcription of genes that code for lipid degrading enzymes, still expression of *Nudt7α* is low in kidney (as judged from mRNA data, see figure 3 in paper I). However, NUDT19 is highly expressed in kidney and may have a similar regulatory role in kidney as NUDT7 in other tissues [59].

The mRNA expression of *Nudt7α* in liver was down regulated by treatment with a PPARα agonist (Wy-14,645) in wild type mice. This regulation was PPARα dependent, since the *Nudt7α* mRNA level in PPARα-/- mice was not affected. The promoter region of *Nudt7α,* at -959 to -971 upstream of the ATG start site, contains a putative peroxisome proliferator responsive element (PPRE) consisting of a direct repeat 1 site (DR1- TGACCTGTGACCT) that potentially can bind the PPARα/RXR heterodimer and in this case repress *Nudt7α'*s expression. The down-regulation in expression during peroxisomal proliferation that follows PPARα activation by agonists may have more profound effects on the activity due to the fact that size and abundance of the organelle increase within the cell, which would further "dilute" the activity in the organelle. This was also found when total diphosphatase activity was measured in isolated liver peroxisomes from clofibrate treated and non-treated mice. Peroxisomal incubations with C_6-CoA and a C_{14}-CoA thioether (a non-hydrolysable molecule for ACOTs) showed that the specific dihosphatase activity decreased about 70% with the medium chain ester, and approximately 30% with the C_{14}-CoA ether. However, no difference was seen during incubation with CoASH. These data supports the mRNA expression data and also indicate that the preferred substrates for NUDT7α are indeed medium chain acyl-CoA esters and not CoASH. In ***paper II*** we analyzed the regulation by fasting, and indeed the expression decreased at mRNA level also during fasting.

The intraperoxisomal CoA pool has been shown to increase during various metabolic conditions, including clofibrate treatment in rat hepatocytes [123]. The expression of most of the known peroxisomally located ACOTs are also known to increase during this condition, probably to support the ongoing β-oxidation at high speed, which requires high amounts of available CoA for the thiolase reaction for the β-oxidation to proceed. A down regulation of NUDT7*α* and thereby a decreased CoA metabolism is in line with this notion.



Picture of NUDT7α and ACOTs during PPARα activation. PPARα signaling would decrease the activity of NUDT7α and thus liberate more substrates for ACOTs and therefore preserve CoA for β-oxidation.

So during "normal" condition the medium chain acyl-CoA activity of NUDT7α would theoretically both regulate the speed of β-oxidation, and also be an important factor to regulate/prevent CoA accumulation in the organelle based on the hypothesis that CoA is co-transported into peroxisomes together with fatty acids. Also, free CoASH is generated in peroxisomes due to the presence of e.g. carnitine acyltransferases, ACOTs and *N*-acyltransferases under "normal" physiological conditions.

The chain-shortened acyl-CoA products of β-oxidation would be too big and bulky molecules to leave the organelle by diffusion and no "exit-transporter" for CoA-esters is still known. However, the action of NUDT7α generates 3',5'-ADP and 4'-phosphopantetheine or 4'-acylphosphopantetheine of which 3',5'ADP is probably transported out from the organelle by the Slc25a17 transporter by counter-exchange with CoA, FAD or NAD^+ [48]. Also 4'-phosphopanteine may be transported by Slc25a17, which just leaves the medium chain 4'-acylphoshopantetheine "behind". Although 4'-acylphoshopantetheine might be small enough to diffuse via PMP22, another possibility is further degradation of these products. We have tested this hypothesis and found that in fact 4'-lauroylphosphopanteteine is a substrate for both the type-1 thioestarese ACOT3 (with a V_{max} of ≈ 1.4 μmol/min/mg and a K_m of ≈ 8 μM) and the type -2 thioesterase ACOT8 (with a V_{max} of ≈ 0.5 μmol/min/mg and a Km of ≈ 8 μM), at least *in vitro* (*Tillander et al*, unpublished data). This finding adds to the possible functions of ACOTs in that they may have important functions in producing metabolites that are small enough to be transported out of peroxisomes via diffusion through the peroxisomal membrane channel comprised of PMP22 (or PXMP2).

4.2 PAPER II

The study ***"Tissue expression and regulation of the 'Pexiome' in the mouse"*** is a transcription based investigation of the genes that codes for peroxisomal proteins that constitute the majority of all peroxisomal proteins known today, from now on referred to as the "Pexiome". For this purpose a custom made Taqman low-density array was designed to quantitate mRNA levels of the chosen genes in 24 mouse tissues.
Due to cost limitations cDNA was synthesised from pooled total RNA samples from individual tissue samples. However, very careful quality tests were done before pooling to exclude degraded RNA or contaminating genomic DNA. Also, several genes were analyzed on individual samples and compared to the corresponding results with the tissue pools to confirm that further analysis of the data with pooled samples was appropriate, which it was judged to be. To take of the whole expression data set into consideration, the tissue expression data were further analyzed by two types of cluster analyses. First, hierarchical clustering analysis and Pearson's correlation analysis were performed to investigate how similar tissues were in their expression of the total Pexiome.

The cluster analysis showed that the six different intestinal tissues all grouped together into a cluster with two sub-clusters for proximal and distal intestine respectively. Also colon tended to cluster based on proximal and distal colon. This is in line with the observation that many of the investigated genes had highest expression in the two proximal parts of the intestine with a declining expression in the distal segments, which quite well follows the expression pattern of PPARα [124].
Another group standing out in this analysis is the "adipose tissue" cluster, which consists of white adipose tissue (WAT), adrenal gland, brown adipose tissue (BAT) and heart. These tissues had in general a high number of "highly" expressed transcripts compared to most other tissues in the analysis (however not as high as liver, gallbladder and kidney), and they also seemed to share a similar gene expression profile.

In the tissue dendrogram 3 "outliers" are standing out, namely kidney, liver/gallbladder and testis. These tissues all have high expression of the majority of investigated genes compared to the other tissues in the analysis. Kidney has high expression of certain "kidney specific" proteins such as e.g. *Dao1* and *Hao3*. Liver together with gallbladder also distinguishes themselves from the other tissues both due to high expression of genes in general but also due to high expression of certain genes in bile acid synthesis and glyoxylate metabolism. Testis is most odd in that many genes seem to have unproportionally high expression in this tissue, like several of the *Pex* genes, certain membrane transporters and carnitine handling proteins. If this is a phenomenon of "bulk storage" of mRNA in messenger ribonucleoprotein particles, or if a high amount of peroxisomes is needed in the tissue (but not in the mature spermatocyte) is still a question. However, comparison of the expression of mouse ACOT9 (in ***paper III***) it is

clear that the correlation between protein and mRNA expression is quite poor in testis, as is the case for several other genes.

Next a partitioning clustering analysis (K-means) was performed using modified ΔCt-values (see material and methods in *paper II*). In this analysis the transcripts are grouped into 7 clusters depending on their expression "shape" rather than expression level (see table below).

Cluster (no. of genes)	**Genes**	**Major metabolic function of proteins in cluster**	**Tissue expression**
1 (34)	*Abcd1, Acaa1a, Acot5, Acot8, Acox3, Agps, Aldh3a2, Ide, Lonp2, Mlstd1, Mlstd2, Nudt19, Paox, Pex 1, 3, 5, 5, 6, 7, 11b, 12, 13, 14, 16, 19, 26, Prdx5, Pxmp3, Serhl, Slc22a21, Slc25a17, Tysnd1, Xdh*	Peroxins, etherphospholipid synthesis	**Ubiquitous** *In general low* (exceptions *Acot5, Slc22a21* which show a very restricted pattern)
2 (19)	*Abcd3, Acnat1, Acot4, Acox1, Amacr, Cat, Crot, Decr2, Ehhadh, Ephx2, Gstk1, Hacl1, Hsd17b4, Nudt12, Nudt7, Pex11a, Phyh, Pxmp2, Scp2*	α-Oxidation, β-oxidation, auxiliary enzymes of lipid degradation	**Ubiquitous - Widespread** *High in liver, kidney and "BAT cluster"*
3 (7)	*Abcd2, Acot6, Crat, Ddo, Ech1, Gnpat, Peci*	Unsaturated fatty acid degradation.	**Widespread - Restricted** *High "BAT cluster" and kidney*
4 (8)	*Acaa1b, Acnat2, Acot12, Acot3, Acox2, Pecr, Pipox, Slc27a2*	Auxiliary enzymes of lipid metabolism	**Widespread - Restricted** *High in kidney and liver*
5 (4)	*Baat, Agxt , Hao1 , Uox*	Glyoxylate, purine and bile acid metabolism	**Restricted** *High in liver*
6 (1)	*Dao1*	D-amino acid metabolism	**Restricted** *High in kidney, some expression in WAT, intestine and brain*
7 (1)	*Hao3*	Long chain α-hydroxy acid degradation	**Restricted** *High in kidney and colon*

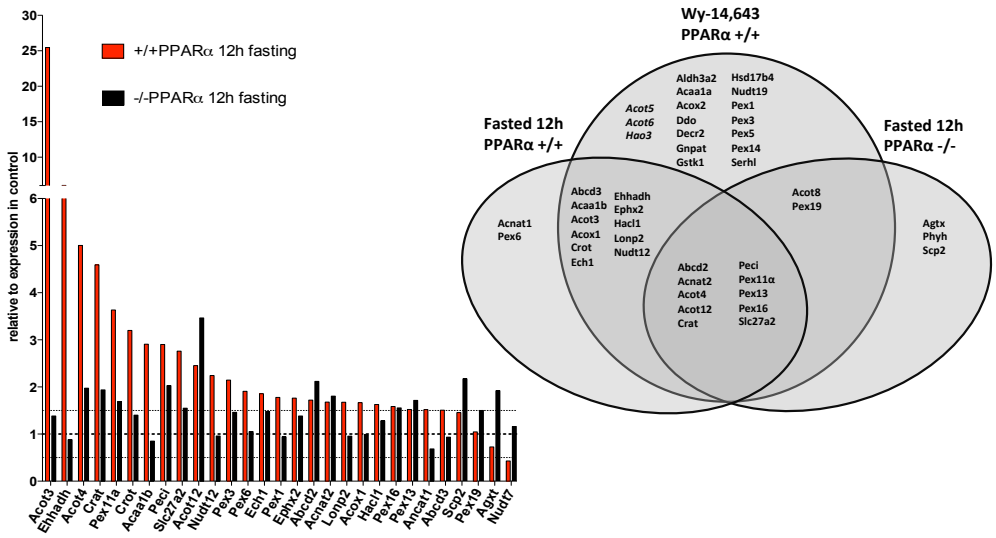
Table that summarize the results from the k-means clustering analysis.

Cluster 1 contain most transcripts investigated in this study, these genes share a widespread tissue expression pattern, however often with a quite low expression level (e.g. the different *Pex* genes and *Acot5*). In this cluster we find all *Pex* genes (except for *Pex11α*), which are (mostly) essential peroxisomal membrane located proteins needed for import of peroxisomal proteins and peroxisome biogenesis and are thus necessary for normal function of the organelle (for review see [125]). Other genes in this cluster are different metabolite transporters such as *Abcd1* and *Slc25a17,* peroxisomal proteases (*Lonp2* and *Tysnd1*) and genes coding for proteins in etherphospholipid synthesis (e.g. *Agps, Mlstd1* and *Mlstd2*)[126-130]. Another common feature of genes in this cluster is a peak in expression in kidney or testis.
Cluster 2 contain many of the transcripts that code for lipid degradation proteins, i.e. most genes involved in straight chain β-oxidation (e.g. *Acox1* and *Ehhadh*), branched chain lipid degradation (e.g. *Phyh, Hacl1, Amacr* and *Scp2*) and auxiliary enzymes of fatty acid metabolism (*Acot4* and *Nudt7*). The clustering of catalase to this cluster is in line with the assumption that this protein would need to be co-expressed with the β-oxidation to cope with the formed H_2O_2 in the first oxidation step of the β-oxidation cycle. Another finding was the co-expression of *Epxh2* and *Pex11α* to peroxisomal

lipid degradation, which might suggest novel functions of these proteins in relation to lipid oxidation.
Cluster 3-7 contain transcripts that have a more restricted tissue expression pattern. In cluster 3 are genes with high expression in BAT/heart/WAT found, in which *Ech1, Peci* and *Abcd2* codes for proteins for unsaturated fatty acid degradation and a predicted transporter thereof [34,131].
Cluster 4 contains transcripts that are highly expressed in both kidney and liver, whereas cluster 5 containing genes are more or less liver specific, e.g. urate oxidase (*Uox*) and bile acid-CoA:amino acid *N*-acyltransferase (*Baat*). *Dao1* (D-amino oxidase) and *Hao3* (α-hydroxyacid oxidase 2) had so peculiar expression patterns that they did not group with any other gene (therefore found alone in cluster 6 and 7 respectively). The high expression of both of these proteins in kidney is well established but the relatively high expression of *Dao1* in WAT and *Hao3* in colon is to the best of our knowledge novel findings that implicate roles for peroxisomes in certain tissues that are still not established.

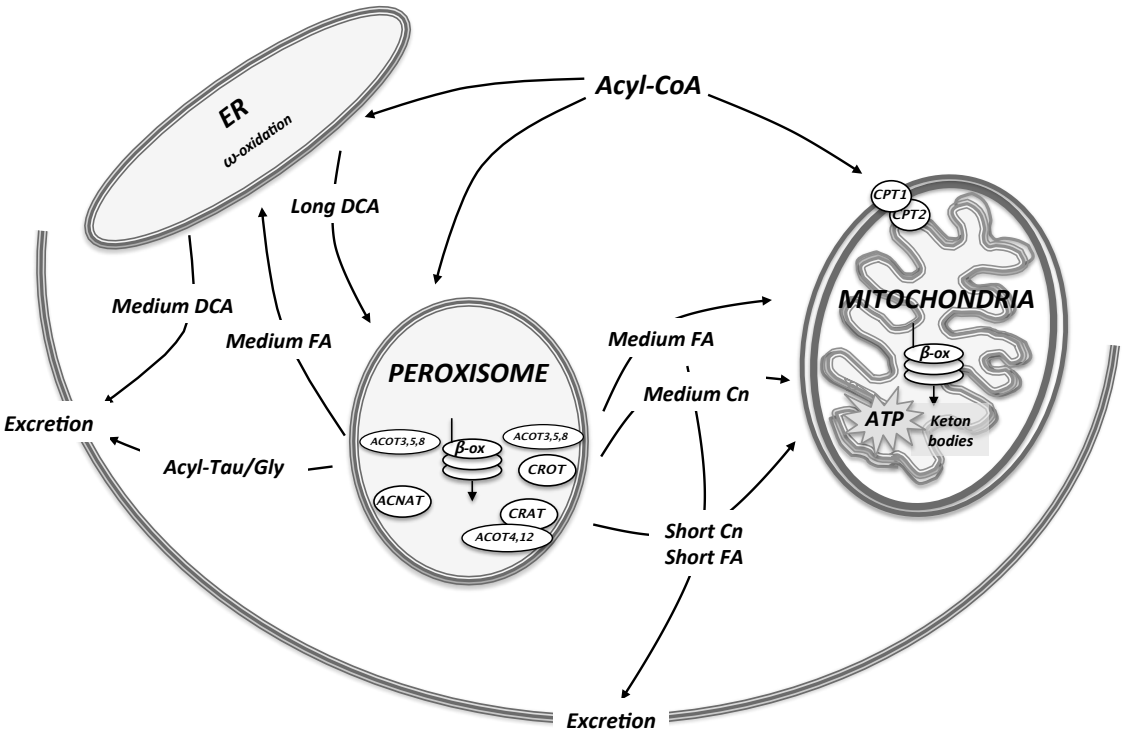
As mentioned above there are several enzymes found in peroxisomes that are active on incoming (substrates) and chain shortened acyl-CoAs (products). This suggests that the organelle might produce different lipid metabolites in a tissue specific manner and during different metabolic conditions. Peroxisomally located ACOTs seem to have quite low expression patterns in general in comparison to NUDT7 and NUDT19, the two carnitine acyltransferases and the taurine conjugating enzymes, which suggests that mainly acylcarnitines and acyltaurines might be major peroxisomal products for further shuttling to the cytosol or mitochondria or for excretion out from the body. However expression of most of these ACOTs is highly increased by PPARα activation (at least in liver). Thus under certain conditions ACOTs will probably generate increased amounts of fatty acids to be released from the peroxisome, but also act a salvage system to maintain an appropriate intraperoxisomal pool of CoA available for ongoing β-oxidation during conditions when the cellular content of long chain acyl-CoA might be high and cause toxic effects, e.g. during fatty acid overload of the mitochondrial (and peroxisomal) β-oxidation system.

PPARα is of major importance during the adaptation to fasting (as described above) and to elucidate PPARα dependency on the regulation of the Pexiome in liver we treated PPARα+/+ and PPARα-/- mice with the PPARα agonist Wy-14,643, or exposed the mice to an overnight fast. As expected we found a “classical” PPARα dependent up regulation in genes coding for straight chain β-oxidation (*Acox1*, *Ehhadh* and *Acaa1b*) together with increased levels of *Acot3* and *Crot* that will generate long chain fatty acid and medium chain carnitine esters, respectively, from the generated β-oxidation products.



To the left: Graph presenting genes that were regulated during fasting in liver from PPARα+/+ or -/- mice. To the right: Picture summarizing the up regulation of genes during fasting and synthetic PPARα activation in liver from PPARα+/+ and -/- mice.

Some genes (e.g. *Acot4,* total *Crat, Peci* and *Pex11α*) were also up regulated in the PPARα-/- mouse during fasting, however the induction was weaker compared to the PPARα+/+ mice. The expression of these genes was also induced by Wy-14,643 treatment in PPARα+/+ mice, but not changed in PPARα-/- mice by this treatment. PPARα expression is also high in kidney, however fasting did not exert the same dramatic induction of genes as seen in liver, further supporting a hypothesis that peroxisomes have different metabolic roles in different tissues. The peroxisomal β-oxidation seems to be of importance for hepatocytes to "rescue" the cell from too high levels of long chain acyl-CoAs and very long-chain fatty acids during conditions when high amounts of non-esterified fatty acids enters the cell (e.g. during fasting) or during insufficient mitochondrial β-oxidation, to produce shorter and/or more soluble compounds for the cell to handle or excrete. This is further supported by the profound dynamic regulation of the Pexiome to e.g. fasting, and for example the strong regulation of the ω-oxidation system in the ER by fasting to produce dicarboxylic acids that are degraded in peroxisomes.



Schematic picture of the fate of acyl-CoAs and products generated from the peroxisome in a hepatic cell. DCA =dicarboxylic acid, FA =fatty acid, Medium Cn =Medium chain carnitine ester, Short Cn =Short chain carnitine ester, acyl-Tau/Gly=Acyl ester Taurine or Glycine conjugated, ER = endoplasmatic reticulum, β-ox =β-oxidation.

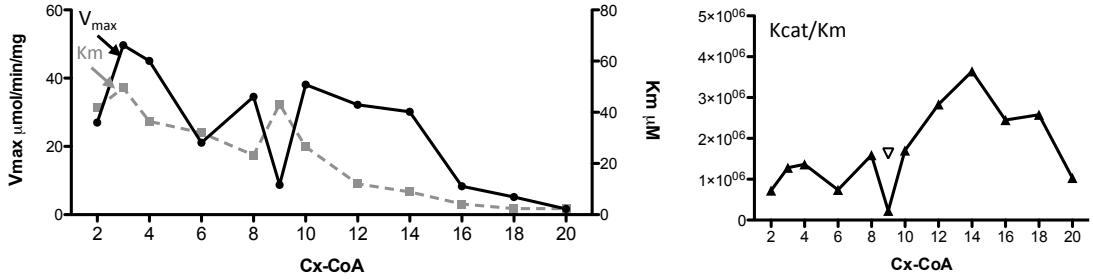
4.3 **PAPER III**

"Acyl-CoA thioesterase 9 (Acot9) in mouse may provide a novel link between fatty acid and amino acid metabolism in mitochondria."

In 1999 Poupon et al. described a novel mitochondrially targeted thioesterase that was identified during a co-precipitation study in an attempt to identify possible binding partners to Esp15, a protein component of plasma membrane clathrin-coated pits that is required for receptor mediated endocytosis. Although the association could not be proved *in vivo*, the group continued to investigate the novel protein. Due to its homology to ACOT7 (at that time called ACT), its approximate molecular mass of 48 kDa and its mitochondrial localisation the protein was named MT-ACT48. MT-ACT48 were stated to be a long chain thioesterase due to the finding of high activity of the protein with C_{14}-CoA and less activity with C_6 -CoA and C_{10}-CoA[81].

In this study we performed a detailed characterization of mouse ACOT9, which showed that the activity of the enzyme is in fact much broader and also harbors activity towards straight medium-chain and short-chain acyl-CoAs as well as to short chain methyl-branched acyl-CoAs.

ACOT9 belongs to the HotDog fold family of proteins and homologous genes are found from bacteria to humans, suggesting a functional conservation through evolution. The *Acot9* gene is located on chromosome X and codes for a protein of 439 amino acids in the mouse. In some species, e.g. human, two variants are produced due to alternative splicing of exon 3, of which one codes for a longer protein of 448 amino acids. Poupon et al. found two transcripts that were 95% identical at nucleotide level with 96% amino acid identity. The other transcript was coded by a second gene, *Acot10,* on chromosome 15, that lacks introns. *Acot10* is a (functional) retrogene only present in the mouse genome and due to its very low expression levels we did not further investigate *Acot10* in this study.
Acot9 showed broad tissue expression in mouse with the highest mRNA expression in testis (that did not correlate well at protein levels when analyzed by Western blot), followed by brown adipose tissue, kidney, lung, white adipose tissue and brain. According to the protein expression ACOT9 is very weakly expressed, or absent, in the intestinal epithelium and in liver.
When screening the activity of recombinant ACOT9 with different straight chain acyl-CoA esters, highest activity (based on V_{max} values) was seen with both long chain acyl-CoA (e.g. with C_{14}-CoA like Popoun et al. previously reported) and short chain acyl-CoA esters, with decreasing K_m values with increasing chain length of the substrates. Very long chain acyl-CoA esters were poor, or not at all, substrates for the enzyme.



To the left: Graph presenting V_{max} (black circles) and K_m (grey squares) of recombinant ACOT9
To the right: Graph presenting K_{cat}/K_m values for acyl-CoAs, in addition is the K_{cat}/K_m value for DMN-CoA shown as a white triangle.

A dip in activity with nonanoyl-CoA (C_9–CoA) was seen for the enzyme, however this acyl-CoA is not a physiological lipid found in metabolism, but its methyl-branched analog dimethylnonanoyl-CoA (DMN-CoA) is. DMN is a metabolite of the peroxisomal degradation of methyl-branched fatty acids like pristanic acid, and DMN is transported to the mitochondria for further metabolism. DMN-CoA was in fact a much better substrate for ACOT9, which suggested that other methyl-branched acyl-CoAs also might be substrates for the enzyme. This hypothesis was tested and ACOT9 was shown to be active preferably with short chain methyl-branched acyl-CoAs, such as isobutyryl- and isovaleryl-CoA. Notably these two molecules are intermediates in the degradation of branched chain amino acids. Other acyl-CoAs that are amino acid intermediates were also tested, however CoA esters with increasing hydrophilic nature (e.g. content of a hydroxyl group), or bulkier structure (benzoyl-CoA) of the CoA ester, decreased the ability of being substrates for ACOT9.

Since ACOT9 was highly expressed in kidney and BAT, and low in liver we isolated mitochondria from these three tissues and investigated the thioesterase activity in these mitochondria with saturated C_2-C_{20}-CoA esters. The thioesterase activities in BAT and kidney mitochondria (in contrast to liver mitochondria) shared a similar activity pattern as the V_{max}-pattern of recombinant ACOT9. C_{14} - CoA thioether is a non-hydrolyzable acyl-CoA for ACOTs due to the thioether bond instead of thioester bound linking the CoA to the fatty acid. This compound potently inhibited the activity of recombinant ACOT9 and was therefore used to elucidate the contribution of ACOT9 to the "crude" activity in the isolated mitochondria. After pre-incubation of the mitochondrial extracts with the C_{14}-CoA thioether, at concentrations tested to inhibit the recombinant ACOT9, resulted in a substantial loss of the similarity pattern previously seen in mitochondria from BAT and kidney, suggesting (but does not prove) that ACOT9 contributes to most of the mitochondrial activity on this CoA esters in BAT and kidney. However, the activity pattern of liver mitochondria was not appreciably affected by this treatment in agreement with the apparent lack of expression of ACOT9 in liver.

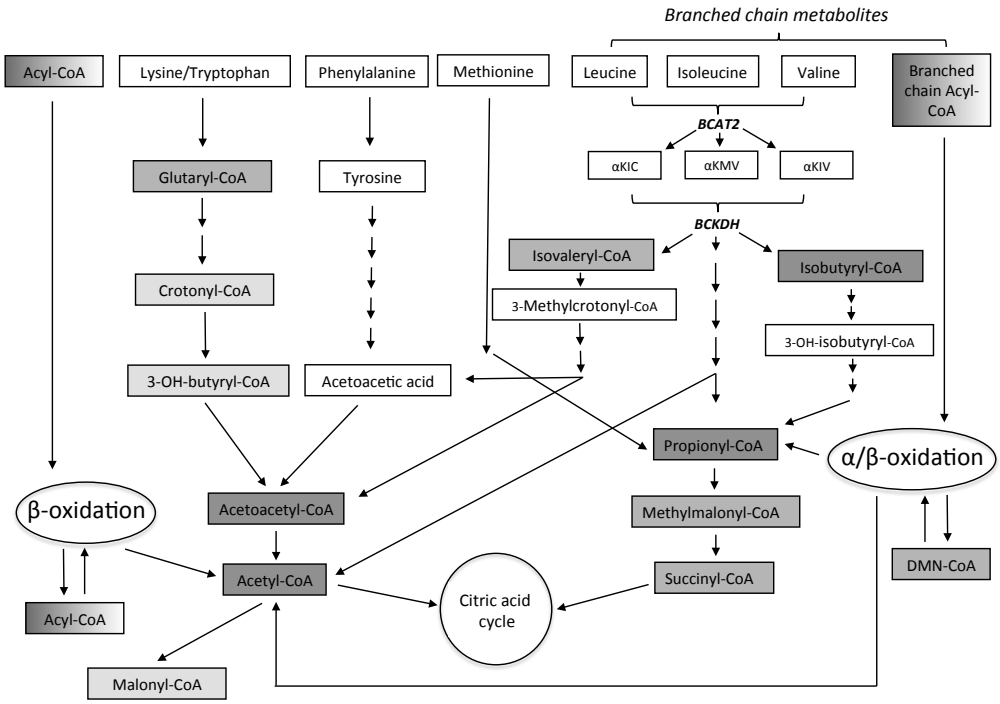
Elevated levels of NADH and CoASH inhibited the enzyme activity both with a short chain substrate (C_3-CoA) as well as the activity with C_{14}-CoA. However, there was a

strong tendency to a "tighter" regulation of the short chain activity than the long chain activity of the enzyme. A similar regulation by NADH on total thioesterase activity was seen also in mitochondria from BAT and kidney, but not in liver mitochondria.
The regulation by NADH is probably allosteric (i.e. an effector, in this case NADH, binds to a proteins allosteric site and thereby affect the activity of the protein) whereas the inhibition by CoASH is more likely competitive due to the fact that the substrate as such contains a CoA-moiety that is likely the major interacting part of the substrate in binding to the active site, and thus free CoASH would compete with other "real substrates" for the binding site. However, it is worth to point out that ACOT8, which is also a CoASH inhibited enzyme, hydrolyzes 4'-acylphosphopantethetiene (see discussion above on *Paper 1*), suggesting that the adenosine moiety in CoA is not needed, or essential for substrate binding and possibly CoASH inhibition and activity.

Overnight fasting down regulated the mRNA of ACOT9 in kidney, and this can be an important function in the mitochondria to provide more short chain acyl-CoAs, such as acetyl-CoA and propionyl-CoA (that after conversion to succinyl-CoA) can enter the citric acid cycle, or be used in gluconeogenesis that also occur in kidney but to a smaller extent than in the liver. The short chain activity may also be important to "rescue" the mitochondria from to high concentrations of short chain acyl-CoA esters that might be produced during pathological conditions. These short chain acyl-CoAs might acts as substrates for CRAT in the mitochondria and with time drain the cells of carnitine (due to the secretion of carnitine esters), and thereby impair long chain fatty acid oxidation by mitochondria.
The higher K_{cat}/K_m values for longer substrates suggest however that the protein mainly would act as a long chain ACOT. But in spite of markedly different K_ms, ACOT9 was in fact able to hydrolyze both short- and long-chain acyl-CoAs simultaneously *in vitro*, suggesting that the short chain activity still might be an important function of ACOT9 during long chain fatty acid oxidation.

Due to the features of the type II ACOTs, ACOT9 probably dimerizes to produce an active site (judged from the results from the sequence analysis), and probably oligomerizes to form trimers of dimers like ACOT7. Early size-exclusion chromatography results of potential homologs to mouse ACOT9 from pig heart mitochondria and hamster and rat BAT mitochondria showed a molecular mass of ≈300 kDa and >240 kDa respectively, which would match this hypothesis [132,133].

Taken together these new findings suggest that ACOT9 may have a regulatory role linking fatty acid and amino acid metabolism in mitochondria, and to be regulated during different metabolic states of the mitochondria.



Schematic picture over substrates for ACOT9 and in which metabolic pathways they can be found. A darker shade of grey represents better substrates for the enzyme.

4.4 PAPER IV

"Fish oil and krill oil supplementation differentially regulate lipid catabolic and synthetic pathways in mice."

A relatively new player on the ω-3 supplement market is krill oil (KO). Krill is a small shrimp-like zooplankton of about 85 species, but the one of major commercial interest is *Euphausia superba,* or Antarctic krill. KO oil is stated to be a safe source of ω-3 PUFAs and to be able to increase the plasma levels of EPA and DHA in humans [134-136]. A difference between the classical fish oil (FO) and KO is in which chemical lipid structure the ω-3 PUFAs are found. In most commercial FOs are the ω-3 fatty acids esterified in TAGs, but this is not the case for KO that instead have most of its ω-3 fatty acids incorporated into phospholipids (or a small amount of non-estrified fatty acids)[137,138]. These structural differences can possibly affect their bioavaibility and their distribution in the body, and thereby may cause different metabolic effects compared to the classical response by FO in supplementary studies.

In this study were the gene regulatory events and lipid modulatory effects of KO and FO investigated when these marine compounds were added (in equal wt% amounts) to a high fat, Western type, diet in C57/Bl6 male mice. Mice were fed the high fat (24% wt/wt) isocaloric diets that mainly consisted of lard in which approximately 6% of the lard was exchanged with the respective marine oil in the two test diets. Even if the two marine oils contain high amounts of ω-3 PUFAs, the FO was more enriched in EPA and DHA so the final FO diet contained approximately double amount of EPA and DHA compared to the KO diet.

During the 6 weeks duration of the study, no significant changes in body weights was detected between the groups (except for a drop at day 37 for the FO group for unknown reasons). Neither could any significant changes be seen in their final body weights, or liver weights, although the liver to body weight ratio tended to be increased in the FO group. Food intake was unfortunately not measured throughout the whole study, but as judged from the measurements in the beginning of the experiment there were no differences in food intake between the groups.

FO decreased plasma cholesterol (free as well as esterified) compared to the control group, which was also reflected in the HDL and VLDL cholesterol content. No changes compared to HF could be seen in the KO group. FO also decreased plasma PL as well as TAG, which was demonstrated by decreased TAG amount in the VLDL fraction. VLDL-TAG appeared also to be decreased in the KO group, however not significantly compared to the control group (HF). It should however be noticed that the post hoc test could not assess any significant differences between the two oil groups in any of these measurements except for the VLDL-cholesterol.
Due to the low amount of plasma was only fatty acid composition of total plasma lipids analyzed. The total amount of ω-3 PUFAs in plasma were very similar between the two marine oil supplemented groups, but it is not known if there were any differences in the

distribution of these fatty acids in the lipid fractions between the groups. The ratio of EPA/DHA in the total plasma fatty acids was very similar to the composition in the diet in both KO and FO groups. The similar EPA/DHA ratio is not surprising since the fatty acid composition of both adipose tissue and plasma quite well reflect the composition of the diet, at least during longer feeding experiments with a stable dietary regime [139]. However, the ratio of EPA/DHA was much lower in liver TAG and PL suggesting that before TAG storage and incorporation into membrane PLs, a substantial amount of EPA is elongated and desaturated to $C_{24:6n-3}$ followed by chain shortening in peroxisomes to DHA (so-called retroconversion) [140]. Interestingly most of the DHA is found in the PL fraction in all three groups.
The incorporation of ω-3 PUFAs decreased the content of many ω-6 PUFAs in plasma, liver TAG and liver PL by KO and in plasma and liver PL by FO. Interestingly KO was more effective in decreasing the amount of arachidonic acid (AA) in plasma and in liver PL, suggesting a more anti-inflammatory role of KO the FO.

Total fatty acid content in the TAG fraction of the liver was increased in FO compared to HF, demonstrating an accumulation of TAG in the livers of animals in the FO group (being significant in the quantitative fatty acid analysis). FO is believed to perform similar actions like fibrates e.g. lowering VLDL secretion and increase plasma clearance of VLDL. This is inline with the reduced amount of VLDL with no change in VLDL particle size seen in this group. However, in spite of increased expression of genes coding for peroxisomal and mitochondrial β-oxidation, and thus likely increased oxidation of lipids this is apparently not sufficient to prevent TAG accumulation in these animals. One hypothesis could be that the capacity to degrade fatty acids is exceeded by a "too high amount" of ω-3 PUFAs in this diet. Long-term intake of high amounts of FO has earlier been shown to cause slight lipoxidative effects in murine livers, and a possible cause of ApoB degradation followed by reduced VLDL secretion from hepatocytes during elevated PUFA exposure has been claimed to be mediated by oxidative lipid species generated from these exogenous PUFAs [141-143]. This accumulation of liver TAG was not seen in the KO group, which instead significantly lowered the amount of NEFA in plasma. Lower amounts of incoming NEFA to the liver might be one reason why TAG accumulation is not seen in the KO group. Another possibility might be the lower and maybe more "preferable" ω-3 PUFA concentration in the diet (if so indicating that to much w-3 PUFAs may be "bad"), or that KO might be protective to lipid peroxidation by the content of the antioxidant Astaxanthin, or by the phospholipid structure itself, which may not down-regulate VLDL secretion as FO.

FO increased the expression of classical PPARα target genes involved in peroxisomal (e.g. *Acox1, Ehhadh, Acaa1b,* and many of the auxiliary enzymes such as most *Acots*) as well as mitochondrial β-oxidation (e.g. *Cpt1, Cpt2,* many of the dehydrogenases and *Acot2*) and ketogenesis (*Hmgcs2*). These effects were not as strong, or were absent, in the group fed KO supplemented diet. Instead KO decreased the expression of multiple genes involved in lipogenesis as well as cholesterol synthesis, like for example HMG-CoA reductase, fatty acid synthase and acetyl-CoA carboxylase A, but without effect on the mRNA expression of SREBP1c. This down regulation was not seen in the FO group. In

fact was FAS activity increased in the FO group compared to KO. This lack of down regulation of lipogenesis (that will use acetyl-CoA generated from β-oxidation) in FO could possibly be an additional explanation to the increased FA levels in the TAG fraction of FO supplemented mice. FO also increased expression of classical PPARα target genes in the intestine, but no major changes could be seen in the KO group.

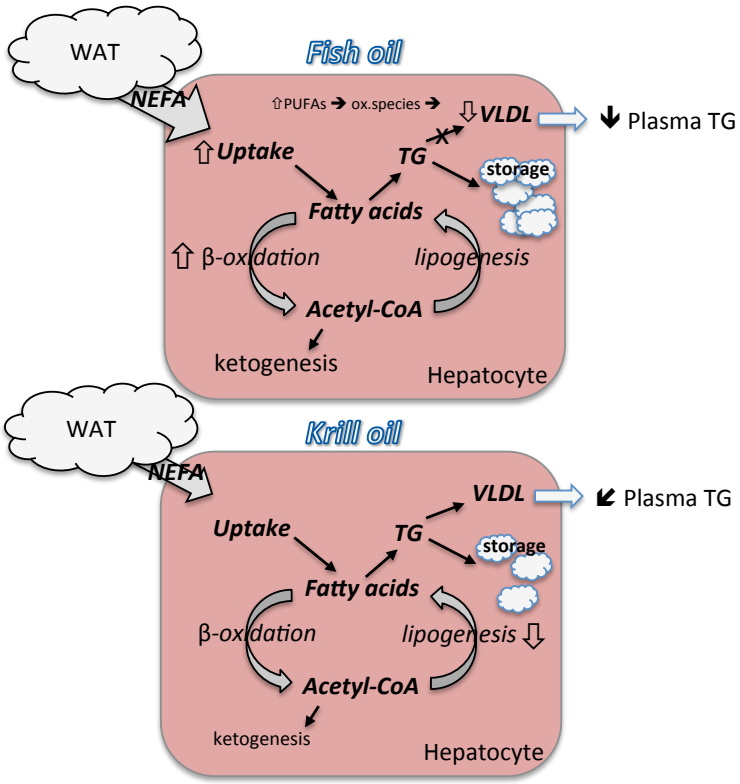


Figure presenting the pathways that seem to be affected by fish oil (top) and krill oil (bottom) based on results from transcriptional regulation data and lipid measurements.

Some of the observations in this study can possibly be ascribed to the different amounts of ω-3 PUFAs in the two diets, however some of the results suggest different gene regulatory mechanisms by KO, not involving PPARα activation which is a well described effect in mice feed with FO.

5 CONCLUDING REMARKS

Paper I. NUD7α is a medium chain diphosphatase that might prevent accumulation of CoASH during normal metabolic conditions in the peroxisome. The enzyme might also contribute to the chain length specificity of products generated by the organelle due to the production of 4'-acylphosphopantetheines that will (probably) not be substrates for further peroxisomal β-oxidation. The down regulation of NUDT7α during increased β-oxidation in the peroxisome (due to PPARα activation) will lead to more available CoASH for the β-oxidation.

Paper II. Most investigated transcripts for peroxisomal proteins are expressed throughout the whole mouse body, however, higher expression in general was seen in liver, kidney, BAT, heart and testis. Metabolic pathways that seem to be expressed in the majority of tissues include; straight chain β-oxidation and the peroxisomal segments of etherphospolipid synthesis, whereas e.g. glyoxlate metabolism and the final steps in bile acid synthesis has restricted tissue expression pattern. This demonstrates the diversity of functions of the organelle. Also the different expression of auxiliary enzymes of β-oxidation suggests that different metabolites will be the produced by peroxisomes in different tissues. Most of the peroxisomal transcriptome is activated during synthetic stimulation of PPARα, and most of the genes regulated by fasting in liver are also PPARα dependent. Regulation of the peroxisomal transcriptome by fasting is much weaker in kidney than in liver.

Paper III. ACOT9 is the only currently identified short chain thioesterase located to the mitochondria. The enzyme is also active with long chain acyl-CoA esters as well as some intermediates and products of branched chain amino acid metabolism. ACOT9 is widely expressed with high expression in e.g. kidney and BAT, but being absent in liver and intestine. Physiological levels of both NADH and free CoA negatively regulate the activity of the enzyme, which suggest rapid regulation of the enzyme during changes in cofactor concentrations in the mitochondria.

Paper IV. Fish oil (FO) and krill oil (KO) supplemented to a high fat diet in equal weight % causes different changes in lipid profiles and gene regulation in mice. FO reduced plasma TAG, PL and cholesterol (free cholesterol and cholesteryl esters). KO only slightly tended to reduce plasma TAG, but reduced levels of NEFA. Similar levels of ω-3 PUFAs were found in plasma and in the PL fraction of livers in both groups, in spite of the different levels of ω-3 PUFAs in the diets. The FO supplemented mice had an increased amount of ω-3 PUFA in liver TAG, in which fraction total FA was markedly increased compared to control. FO showed a classical PPARα activation response by up regulating genes for fatty acid utilization and oxidation, whereas KO down regulates genes in cholesterol and fatty acid synthesis.

6 FUTURE PERSPECTIVES

Further investigations on the interplay between NUDT7α and different peroxisomal ACOTs would be desired to further establish their role in CoA homeostasis and their possible role as regulators for the peroxisomal β-oxidation system. To answer the question if the produced 4'-acylphosphopantetheines are able to pass the peroxisomal membrane or if the ACOTs are needed for further degradation would possibly be investigated using siRNA systems. Also further investigations on what kind of metabolites that are actually products from the peroxisome in the different tissues and during different metabolic conditions would also be of major interest. This would yield further insights into the pathological events that occur in different peroxisomal diseases, but may also highlight the role of the organelle during other pathological and non-pathological conditions. siRNA systems in different cell lines (and possibly also in primary mouse/human hepatocytes) would be a platform to start with, and also investigate products from isolated tissue peroxisomes and their enzyme activity in different *in vitro* assays.

As mentioned in *PaperIII* the human version of ACOT9 is expressed in two splice variants. Further kinetic characterization of the two human splice variants, their transcriptional regulation and what metabolites that will accumulate during deletion or induction of the enzymes could be investigated in different cell lines and also possibly in fibroblasts from patients with different inborn metabolic diseases of mitochondrial lipid and amino acid metabolism.

Further investigations are needed to evaluate the mechanisms behind the different effects seen when mice are given fish oil or krill oil. In this study it is possible that some of the differences is caused by the different amounts of ω-3 PUFAs in the different diets. However probably it does not explain all differences observed. First, the question is if absorption is different between the two oils because of their different molecular properties (TAG vs. PL and free fatty acids). Next, it would be interesting to investigate in which tissues (and lipid fractions), fatty acids from dietary TAG vs. PL end up in, e.g. how does these supplementations change the FA composition of adipose tissue or the brain. An ω-3 PUFA-balanced study further needs to be done to find out if the lack of PPARα induction in krill oil is a due to a dose difference of the ω-3 PUFAs or not, and which other nuclear transcription factors that possibly are regulated in liver and in peripheral tissues by these two marine oils.

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